FLAXSEED OIL INDUCES APOPTOSIS IN THE AGGRESSIVE MURINE MELANOMA CELL LINE B16-BL6

by

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Abstract

Flaxseed is classified as a functional food and is renowned for its exceptional nutrional value. These foods have the ability to either promote overall well-being and/or reduce the risk of certain diseases. Flaxseed, in the form of seed-derived oil, is the highest plant source of the omega-3 fatty acid, alpha linolenic acid. This nutraceutical has been credited with providing protective benefits against breast, colon and prostate cancers. Humans are unable to synthesize omega-3 fatty acids within the body and therefore must obtain sufficient amounts of this antioxidant through diet alone.

Currently, the Western diet is grossly deficient in omega-3 fatty acids and this is in part due to the overwhelming presence of omega-6 fatty acids used in food processing and manufacturing techniques. As one of the highest sources of omega-3 fatty acids, flaxseed is often taken as a supplement to help balance the ratio of omega-3 to omega-6 fatty acids in Western cultures. Although flaxseed has been beneficial in alleviating certain symptoms in patients suffering from cardiovascular disease, diabetes, and cancer, the underlying mechanisms remain unclear. Previous animal studies have shown that a diet supplemented with 10% flaxseed, significantly decreased tumour growth in rodent cancer models. Our studies include the treatment of malignant cells with flaxseed oil in an *in vitro* model. We have shown that flaxseed oil has the ability to reduce cell growth in B16-BL6 cells, an aggressive murine melanoma. Furthermore, a total of seven different oils containing high concentrations of omega fatty acids, including flaxseed oil, olive, sunflower, canola, sesame, peanut and grapeseed, were characterized by HPLC and GC/MS analysis for fatty acid profiles, and these oils were also used to treat B16-BL6 cells. Although all seven oils contain various amounts of omega-3, -6 or -9 fatty acids,

only treatment with flaxseed oil decreased the growth of the aggressive murine melanoma cell line B16-BL6. DNA laddering, acridine-orange staining, TUNEL staining, and FACS analysis using Annexin V and propidium iodide, showed that the flaxseed-treated cells were undergoing apoptosis, a type of cell suicide. Therefore, due to its ability to selectively inhibit malignant cell proliferation, flaxseed oil has significant potential as an anti-cancer therapeutic.

Keywords

Flaxseed, omega-fatty acids, cancer, natural products, anti-cancer therapeutics

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And finally, I dedicate this chapter of my life to my beautiful baby girl Abby. You are the reason I decided to push myself to complete this thesis. Your early arrival gave me the confidence and courage to stand in front of my peers and defend this thesis. I promise to support you in all that you do and I will try my best to make all your wishes come true.

Table of Contents

List of Figures

List of Tables

List of Appendices

Abbreviations

AA	arachidonic acid
ALA	alpha linolenic acid
APS	ammonium persulfate
BSA	bovine serum albumin
^{o}C	degree Celsius
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DMEM	Dulbecco's Modified Essential Medium
EC	epicatechin
ECG	epicatechin gallate
ECL	enhanced chemiluminesence
ED	enterodiol
EDA	eicosadienoic acid
EDTA	ethylenediamine tetra acetate
EFA	essential fatty acid
EPA	eicosapentaenoic acid
ET	enterolactone
FACS	fluorescence-activated cell sorting
FAME	fatty acid methyl ester
FBS	fetal bovine serum
GC/MS	gas chromatography/mass spectrometry
GLA	gamma linolenic acid

hrs	hours
LA	linoleic acid
M	molarity – moles/litre
mg/ml	milligrams per millilitre
min	minute
mm	millimeter
nm	nanometer
ml	millilitre
PARP	polyadenosine diphosphate ribose polymerase
PBS	phosphate-buffered saline
PI	propidium iodide
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
tR	retention time
RPM	revolution per minute
SDS	sodium dodecyl sulphate
SDG	secoisolariciresinol diglucoside
sec	second
SIRS	systemic inflammatory response syndrome
TAG	triacylglycerols
TBS	tris-buffered saline
TBST	tris buffered saline with 0.1% Tween 20
TdT	terminal deoxynucleotidyl transferase

TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
µg	microgram
µl	microlitre

1.0 Introduction

Traditionally, natural products were used as medicine by indigenous populations to treat a broad range of diseases and ailments[1,2]. In developing countries, the majority of people still rely on the use of traditional medicine and the use of plant sources for treatment as their primary healthcare[3]. This translates into approximately 3.5 to 4 billion people worldwide[3]. These traditional ways have piqued the interest of contemporary researchers and have encouraged in-depth analysis of plant materials commonly found as part of these native treatments. Historically, natural products have always provided a starting point and pharmaceutical companies have utilized plant extracts to produce crude therapeutic formulations[4]. In recent years, natural products, in their naturally occurring state, have been at the forefront of drug discovery experiments. Furthermore, the chemical diversity of natural products has led researchers to isolate and screen plant materials in hope of finding compounds that may possess medicinal properties[4,5].

Although many therapeutic areas have reaped the benefits of natural products and drug development, oncology has had the most success. Approximately 60% of the new drugs intended to treat cancer were developed from a natural product source[5-7]. Between 2005 and 2010 alone, there have been a total of 19 natural product-based drugs which have been approved for marketing worldwide[5]. In addition, many of the leading antitumor drugs currently in use were developed from a natural product source[5,6]. One of the best recognized of these anti-tumour drugs is Paclitaxel (Taxol®). Ironically, *Taxus brevifolia* Nutt. *(T. brevifolia)*, commonly known as the Pacific Yew, was once referred to as the tree of death because of its toxicity to humans and wildlife, is now referred to as the tree of life for many cancer patients since the discovery that taxol was present in its

bark[7,8]. Paclitaxel, a mitotic inhibitor originally isolated from *T. brevifolia* but now synthesized chemically from more readily available precursors is used in the treatment of various cancers, including, lung, ovarian and breast cancer[7-10]. Paclitaxel stabilizes microtubules in rapidly dividing cancer cells and interferes with the normal breakdown of microtubules during cell division[1,7-10]. Although it may be the most recognized example of a natural product used in modern medicine, many drugs have been derived from a natural product source.

1.1 Traditional Use of Natural Products in Medicine

Natural products are defined as chemical compounds or substances produced by a living organism or found in nature. These compounds often have chemical properties that are beneficial for use in pharmaceutical drug discovery[11]. A chemical compound can be classified as a natural product regardless if it can be prepared by total synthesis" [11]. Natural selection and biological interactions have evolved the chemical diversity of naturally occurring compounds, allowing them to modulate or influence certain biological functions[7,8]. The affinity and specificity that these natural occurring compounds may have with biological macromolecules make them an ideal starting point for drug discovery[4-8].

Historically, the medicinal use of natural products by humans has been shown to go back to the time of Neanderthals. A palaeoanthropological study at the cave site of Shanidar, located in the Zagros Mountains of Kurdistan in Iraq, discovered pollen deposited within the graves of a burial sanctuary[9]. These findings suggest that Neanderthals may have used certain plant materials for medicinal purposes over 60,000

years ago[9]. Before the development of modern medicine, our ancestors were forced to depend solely on natural products for the treatments of ailments, as well as aches and pains. Whether it be, to chew on herbs to relieve pain, to wrap leaves around wounds to prevent infections, or to create a paste from a mixture of plant materials to encourage faster healing, the natural remedies that have been passed down through generations are now grabbing the attention of researchers for their potential benefit to modern medicine[1,10].

It is often difficult for researchers to explain how naturally occurring plant compounds can have biological effects in humans. One commonly accepted hypothesis includes coevolution of biological systems. Interacting organisms that have evolved alongside each other often develop compounds that can interact with the biological processes of one another[12]. The result is an increased likelihood of survival. It is survival of the fittest, and as these compounds evolve and become more advantageous to the host species, natural selection will allow for these organisms to survive[12]. Some examples of coevolution and natural products include the toxins that plants have evolved to protect themselves from herbivores[12]. These toxins are now isolated and used in the manufacture of laxatives, and used for the treatment of constipation in humans[12].

There are numerous natural products that are known to induce apoptosis, and these compounds which regulate apoptosis are of high medical significance, as cell death evasion is a hallmark characteristic of malignancy. Apoptolidin, a macrolide originally isolated from *Amicolatopsis* sp., is an example of a natural product that selectively targets mitochondria and inhibits ATP enzymatic functions in several cancer cell lines[13]. Since mitochondria are the energy-producing organelles of the cell, natural products that can

selectively target mitochondrial functions in malignant cells are of high importance[14]. In recent years, there has been an increase in studies that show that many of the common herbs and vitamins that are readily available to consumers may in fact affect mitochondrial activity, directly or indirectly[14]. With many cancer patients taking vitamins and supplements in conjunction with, or instead of chemotherapeutic- and radiation-treatment plans, it is becoming more urgent to understand the chemical interactions that these natural products may have on malignancies, both in the presence and absence of prescribed anticancer drugs[14]. The potential of herb and nutrient supplement interactions with drugs have caused many researchers to systematically evaluate the potential of "functional foods" in treatment plans.

1.2 Flaxseed Oil Consumption

Flax, in the form of seeds or seed-derived oil, is a “functional food” recognized for its exceptional nutritional value due to its high concentration of fiber-based lignans and large amounts of omega fatty acids[15-17]. Flaxseed is one of the most important oilseed crops, as every part of the plant can be used commercially[15-16]. Canada is the major exporter of flaxseed and flaxseed products, contributing about 80% of the global trade supply[15]. *Linum usitatissimum* L., a member of the Linaceae family and commonly referred to as flaxseed, has been cultivated for food and fibers for over 30 000 years. It is used in the production of paints and textiles, where the seeds are crushed for the extraction of oil[15-16]. *Linum usitatissimum* is referred to as flaxseed or linseed, depending on whether the flax is to be used for human consumption, or for industrial purposes, respectively[15,16]. Flaxseed is unique among oilseeds because its oil is a source of the

essential omega-3 fatty acid, alpha linolenic acid (ALA)[15,16,18]. It is critical that humans incorporate foods high in ALA into their diets because the human body lacks the required desaturation enzymes that are necessary for inserting double bonds at the 12- and 15-carbons[18-20]. Following ingestion, ALA is metabolically converted into the longer chain omega-3 fatty acids, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA)[18-20]. It is crucial to obtain sufficient amounts of EPA and DHA from the diet, since these two omega-3 fatty acids can partially reduce the pro-inflammatory effects of the omega-6 fatty acid arachidonic acid[18-20]. Since flaxseed oil is recognized as one of the largest sources of ALA, it has also been titled as a health promoting, functional food[16].

Many studies focused around flaxseed consumption have shown increased health benefits to patients suffering from symptoms caused by cardiovascular disease[17], diabetes[21], and cancer[22]. Unfortunately, the underlying mechanisms of how flax seeds or flaxseed oil, influences biological processes remains a mystery. Unsaturated fatty acids have previously been shown to modulate a number of lymphocyte functions[14,23]. These functions include natural killer cell activity, cytokine release, adhesion molecule expression, as well as cell proliferation and cell death[14,23]. The majority of studies focused around flax seeds and flaxseed oil have been investigated in animal models. Previous animal studies have shown that a diet supplemented with 10% flaxseed, significantly decreased tumour growth in rodent cancer models[23-27]. In contrast, our studies focus on the direct exposure of malignant cells to flaxseed oil in an *in vitro* model. The antioxidant properties of flaxseed oil also make it a favourable supplement that is often included in established treatment regimens for patients undergoing chemotherapy and radiation therapies. By understanding how flaxseed oil interacts with

malignant cells, both in the presence and absence of chemotherapies, there is a potential to develop an anticancer drug or drug combination that may have minimal side effects.

1.3 Functional Foods, Flaxseed and Fatty Acids

The increased consciousness of the general population regarding healthier lifestyles and increased awareness of what they are consuming, has gradually changed the perception of foods as a physical requirement to the possibility of using foods as contributing factors to overall health and wellbeing[29-32]. Hence, functional foods are described as foods that provide basic nutritional requirements but also, have additional known health-promoting benefits[31,32]. Functional foods tend to have one of two effects: they either enhance physiological wellness, or they reduce the risk of a particular disease[31,32]. The concept of functional foods has been previously investigated and both nutritional and epidemiological studies have shown that poor diets can cause or influence chronic diseases, including cardiovascular disease, diabetes and cancer[29-32]. Although the term functional food is commonly used, there are few regulations around this category. Some of the most popular functional foods are foods that are high in antioxidants, polyphenols, prebiotics and probiotics[31,32].

Flaxseed, in the form of seeds and seed-derived oil, is an example of one of these known functional foods. The oil is a rich source of the omega-3 fatty acid, alpha linolenic acid, a fatty acid that is often lacking in Western diets. Flaxseed and in particular omega-3 fatty acids, have been shown to have protective benefits against breast, colon and prostate cancers[22]. Furthermore, studies have shown that patients who introduce flaxseed into their diets demonstrated signs of resistance to early stages of

some cancers[33], reduced the risk of hormone-related cancers[26,34], and diminished the rate of metastases of some breast cancers[25].

1.4 Omega-3 and Omega-6 Structures, Classification and Desaturation

Although the exact mechanisms to explain how flaxseed oil affects cancer rates and metastases are not known, many suggest that establishing an appropriate balance of omega-3 to omega-6 fatty acids may in part be responsible[18,35]. Both omega-6 and omega-3 fatty acids are required for proper cellular function and human development[18,35]. Unlike some fatty acids that are capable of being synthesized within the human body, omega-3 alpha linolenic acid and omega-6 linoleic acid are considered essential and must be acquired from our diets[18,22,35,36]. However, humans do possess the required enzymes that allow for elongation of alpha linolenic and linoleic acid and their conversion to the longer chain counterparts, docosahexaenoic acid and arachidonic acid[18,22,35,36]. This conversion is inefficient and the rate of synthesis is incapable of sustaining daily requirements[35]. Fatty acids are mainly present in the body as triacylglycerols, a complex that is formed by three fatty acids attached to a glycerol backbone[18,35]. Although fatty acids can vary from short two carbon chains to as long as eighty carbon chains, the most common lengths found in foods tend to be between fourteen and twenty-two carbon atoms[18,35]. A saturated fatty acid chain is one that contain only a single carbon-carbon bond at each position, which results in a chain linked to the maximum number of hydrogen atoms[18,35]. If the chain contains at least one double bond, then the fatty acid is called unsaturated. Polyunsaturated fatty acids possess multiple double bonds between carbon atoms[18,35]. The positioning of the double bonds and the position of its hydrogen

atoms determines the structure of the fatty acid[18,35]. If at the double bond both hydrogen atoms are positioned on the same side of the carbon chain, then it is described as a *cis* fatty acid[18,35]. However, if the hydrogen atoms are positioned on opposite sides of the double bond, then the fatty acid is described as *trans*[18,35]. Cis configurations tend to be the most popular in unprocessed foods, whereas *trans* configurations are common in ruminant meats and milk, as well as in unsaturated oils that have undergone hydrogenation[18,35].

Omega fatty acids are further classified and grouped into fatty acid families by the number of carbon atoms within the chain, and most importantly by the placement of the first double bond[37]. If the first double bond is located between the third and fourth bond position from the methyl terminal end, then the fatty acid is classified an omega-3 fatty acid[37] (Figure 1). In the same sense, if the first double bond is placed between the sixth and seventh bond position from the methyl terminal end, then the fatty acid is classified as an omega-6 fatty acid[37] (Figure 1). Docosahexaenoic acid (22:6 ω-3) is therefore a 22 carbon chain with 6 double bonds, with the first double bond situated at the third-fourth carbon position from the methyl terminal end, and is hence an omega-3 fatty acid[37]. Arachidonic acid (20:4 ω-6) is a 20 carbon long chain containing 4 double bonds, with the first double bond located at the sixth-seventh carbon bond position from the methyl terminal end, and hence therefore an omega-6 fatty acid[37]. The metabolic pathways that describe the synthesis of omega-3 and omega-6 fatty acids are similar, with both consisting of desaturation and elongation steps[18,35,37]. The byproduct of each step changes dramatically depending on which class of omega fatty acids commences the pathway.

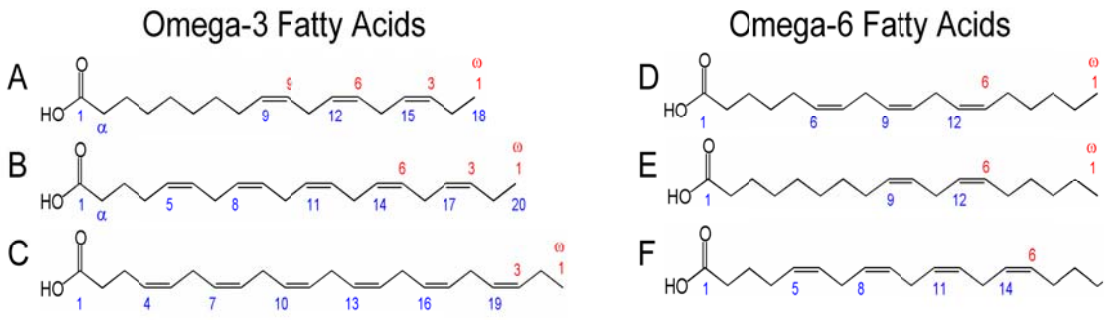

Figure 1 Chemical structures of the omega-3 and omega-6 fatty acids (A: Alpha Linolenic Acid, B: Eicosapentaenoic Acid, C: Docosahexaenoic Acid, D: Linoleic Acid, E: Gamma Linoleic Acid, F: Arachidonic Acid) [38]

Omega fatty acids are forced to compete with one another for available desaturation enzymes (Figure 2), and with a diet heavily concentrated with omega-6 fatty acids, most Western populations tend to have a largely improperly balanced ratio that favours omega-6 fatty acids[18,35,37]. The ratio can be rather dramatic from one population of the world to another. If we compare levels of the omega-6 fatty acid, arachidonic acid, to the omega-3 fatty acid, docosahexaenoic acid, the ratio in the Western population (meat and dairy rich diets) averages 46:1, while the Eastern population (fish and vegetable-rich diets) averages is 1.3:1[39]. This difference may be part of the explanation for elevated levels of many cancers and increased heart disease in Western populations as compared to Asian. The American Heart Association recommends an omega-6 to omega-3 fatty acid ratio of 10:1 [39].

This offset is most influential on docosahexaenoic acid and arachidonic acid, since they are direct competitors for desaturation enzymes in the omega fatty acids metabolism pathway[18,35]. The Δ6- and Δ5-desaturase enzymes are two examples where this direct competition is present. Δ6-desaturase is the primary enzyme that

metabolically converts the omega-6 fatty acid linoleic acid to dihomo-γ-linolenic acid via γ-linolenic acid, while its competing omega-3 fatty acid, alpha linolenic acid is converted to eicosatetranoic acid, via octadecatetraenoic acid[18,35]. The Δ5-desaturase enzymes metabolically convert the omega-6 fatty acid dihomo-γ-linolenic acid to its end product arachidonic acid, while promoting conversion of the omega-3 fatty acid eicosatetraenoic acid to both eicosapentaenoic acid and docosapentaenoic acid[18,35]. Docosahexaenoic acid, the end product of the omega-3 fatty acid desaturation pathway, is metabolically converted from docosapentaenoic acid through two independent pathways using Δ4-desaturase, or Δ7-elongase and the additional Sprecher pathway [18,35].

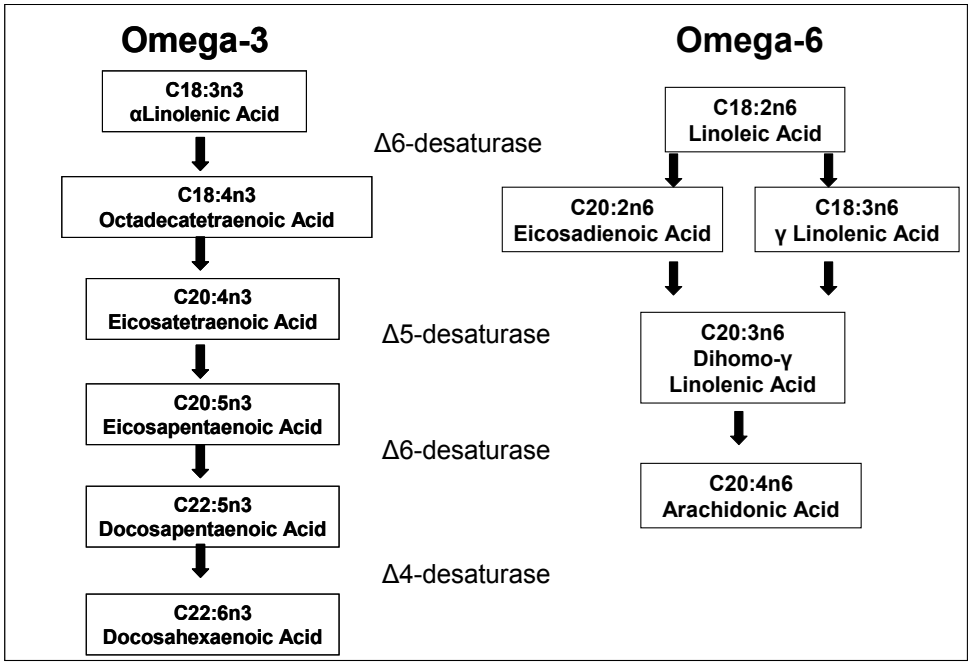

Figure 2 Competing desaturation and elongation pathway of the omega-3 and omega-6 fatty acids[20].

1.5 Omega-3 versus Omega-6 Functions

The metabolites from both the omega-3 and the omega-6 pathways have several functions. They can influence cell viability and functionality through eicosanoid regulation, receptor interactions, membrane fluidity and/or gene expression[40]. Arachidonic acid and eicosapentaenoic acid are both strong regulators of various families of eicosanoids, a family of diverse hormone-like agents that include prostaglandins, prostacyclins, leukotrienes and thromboxanes[35]. The various eicosanoids can act as signaling molecules with specific receptor-signaling cascades that can have rapid and profound effects on physiology and immune functions. Eicosanoids derived from omega-6 fatty acids tend to be pro-inflammatory, whereas eicosanoids derived from omega-3 fatty acids tend to be anti-inflammatory[35,40]. Arachidonic acid derived thromboxane TXB2 and eicosapentaenoic acid derived thromboxane TXB3 are just one example of the difference of eicosanoid functions derived from omega-6 versus omega-3 fatty acids. TXB2 is a vasoconstrictor and platelet activator through TP-α receptors. In contrast, TXB3 impedes TXB2 functions by inhibiting TXB2-mediated platelet aggregation, and increasing vasodilation[40].

Fatty acids can also be major components of cellular membranes. Membrane fluidity is highly influenced by the presence of omega-3 or omega-6 fatty acids. This is due in part to the large structural differences in the acyl chains between the two PUFA families[40]. Membrane fluidity can greatly affect cellular functions as it affects membrane permeability, and secondarily protein trafficking. Researchers have previously shown that the omega-3 fatty acid, docosahexaenoic acid, regulates phosphotidylserine by signalling it to accumulate[40]. The accumulation of phosphotidylserines in the cytosolic

side of the plasma membrane can facilitate stimulation and membrane translocation of certain protein kinases[40]. Akt is an example of one of the protein kinases that can be activated by accumulation of cytosolic leaflet phosphotidylserines and results in an enhanced cell survival signal[41]. Since Akt/PI3k is a pro-survival pathway, the accumulation of Akt at the cellular membrane may in fact leave mitochondria vulnerable to proapoptotic ligands. Fukui *et al.* (2013)[41] have previously shown that omega 3 fatty acids are capable of causing mitochondrial reactive oxygen species (ROS) accumulation in human breast cancer cells. The accumulation of ROS in turn activates apoptosis through Caspase 8[41]. Both the intrinsic and extrinsic apoptotic pathways crosstalk through Caspase 8[41].

In vitro studies have been performed to measure prostate cancer cell growth in the presence of omega-3 and omega-6 fatty acids[39]. Researchers found that treatment with omega-3 fatty acids inhibited prostate tumourigenesis in tissue culture models, while the cells treated with omega-6 fatty acids showed increased rates of cellular proliferation[39]. These findings are not unique to prostate cancers, and researchers have repeated these studies in breast cancer cells as well. In animal studies, mice fed diets high in omega-6 fatty acids, primarily from corn and safflower oils, had increased mammary tumour proliferation and higher rates of metastases[42-44], while the mice fed diets high in omega-3 fatty acids primarily from fish oils, showed lower rates of metastases and decreased mammary cell proliferation[42-44]. Natural products that are capable of interfering with cell proliferation or inducing apoptosis are of high importance in oncologic research since they promote a means to interfere with tumour growth.

1.6 Natural Products, Cell Growth and Cell Death

It is well known that many of the compounds that are currently used to treat human diseases are derived from plant, microorganisms or animal sources[1]. With over 10 million new cases of cancer emerging yearly, it is the second leading cause of death in North America[1]. Oncology is one field that could benefit from better treatments as well as preventive options[1]. Drug discovery from medicinal plants has already played a critical role in the treatment of cancer and approximately 60% of the drugs currently being used in the field of cancer were developed from a natural source[5]. Cancer treatments usually regulate tumour growth in two specific ways based on the hallmark characteristics of cancer. These include interference with cell growth and increased susceptibility to cell death. The normal cell cycle is regulated by increases and decreases in specific cyclin levels in a tightly regulated manner[45,46]. The relative change in cyclin levels ensures proper DNA replication, and consequently cellular division. There are four phases of the cell cycle required for cell proliferation: G1, S, M and G2[45,46]. In S phase, the DNA is replicated, while in M phase the newly-replicated DNA is segregated, and the cell physiologically divides into two daughter cells. These two phases are separated by gap phases referred to as G1 and G2[45,46]. Cells can also withdraw into a third gap phase referred to as G0, where cell cycle division stops. This third gap phase can be a reversible state, and usually occurs as a result of high cell density, or a lack in nutrient availability in cultured cells[45,46]. In addition, cells can enter an irreversible state of withdrawal from the cell cycle[45,46]. This state occurs in response to terminal differentiation or senescence[45,46]. The cyclin-dependent kinases and the cyclins control the cell cycle and ensure regulated progression through the cell cycle[45,46]. The transition

of one phase to another is monitored by checkpoints. If the normal progression through the cell cycle is detected as incorrect by a mechanism sensor at the checkpoint, the cell is inhibited from progressing through the cycle to the next phase, and remains arrested until the checkpoint signal is corrected[45,46]. Malignancies are a result of mutations in somatic cells and mutated genes tend to directly or indirectly affect the cell cycle[45]. For normal cells, mitogenic stimulation is required for the cell to enter the cell cycle[45,46]. However malignant cells frequently have constitutively active growth signaling pathways (oncogene activation) and are able to enter and remain in cycle permanently, consequently resulting in the formation of tumours[45]. Furthermore, malignant cells are often plagued with faulty DNA repair and ineffective checkpoints which do not prevent proliferation of damaged or defective cells[45]. These malfunctions intensify the genetic instability and encourage a vicious cycle of uncontrolled cell proliferation[45]. Therefore, targeting deregulated components in the cell cycle by pharmacological treatment is one ideal strategy in cancer therapies[45]. Antineoplastic agents are drugs that are designed to target and inhibit the cell cycle by disrupting the necessary mechanisms for the formation of mitotic spindles, and for the replication of DNA and regulation of the nucleotide synthesis machinery[45,46]. Treatment regimens for patients undergoing chemotherapies tend to include combinations of drugs designed to target different stages of the cell cycle. There is an additive anti-tumour benefit of multi-targeted cell cycle chemotherapeutic agents, and as long as the apoptotic machinery is functional, the cell is forced to correct its cycle, or to initiate apoptosis[46].

The evasion of cell death, or apoptosis a type of cell suicide, is a second hallmark characteristic of cancer. On average, each person has 50 billion cells that die each day.

In one year, it is suggested that the average person proliferates and destroys a mass of cells equivalent to their body size[47]. It is no longer surprising to see the link between cell death evasion and the development of cellular hyperplasia, and consequently malignancy. Furthermore, the exposure to known, as well as currently un-identified carcinogens is becoming increasingly common from the environment. Carcinogens are known to cause cancer and enable these transformed cells to evade apoptosis, in turn promoting tumourigenesis[47]. There are two pathways in mammals and other vertebrates that control apoptosis: the intrinsic pathway which is regulated by the Bcl-2 family of proteins and primarily affects mitochondrial function, and the extrinsic pathway that requires an external signal transduced through a death receptor of the TNFα receptor family[47-49] (Figure 3). In the intrinsic pathway, the Bcl-2 proteins are activated in response to intracellular stimuli[47-49]. These stimuli can range from developmental cues, cytotoxic stress, nutrient and growth factor deprivation, infection, DNA damage, and hypoxia[47-49]. These stimuli disrupt proapoptotic Bcl-2 proteins found in the mitochondrial membranes, causing increased mitochondrial membrane permeabilization and the release of intramembrane proteins, including cytochrome C and Smac/DIABLO[50]. The release of these proteins into the cytosol, promotes an activation cascade called the apoptotic pathway. In particular the release of cytochrome c allows the formation of a structure called the apoptosome complex that includes Apaf-1 and caspase-9[49]. The newly formed apoptosome complex activates caspase-3 which is a wide specificity protease frequently thought of as the "executioner"[49]. Following activation, caspase-3 cleaves and proteolytically degrades a host of intracellular proteins, and ultimately produces cellular and biochemical changes necessary for apoptosis[49].

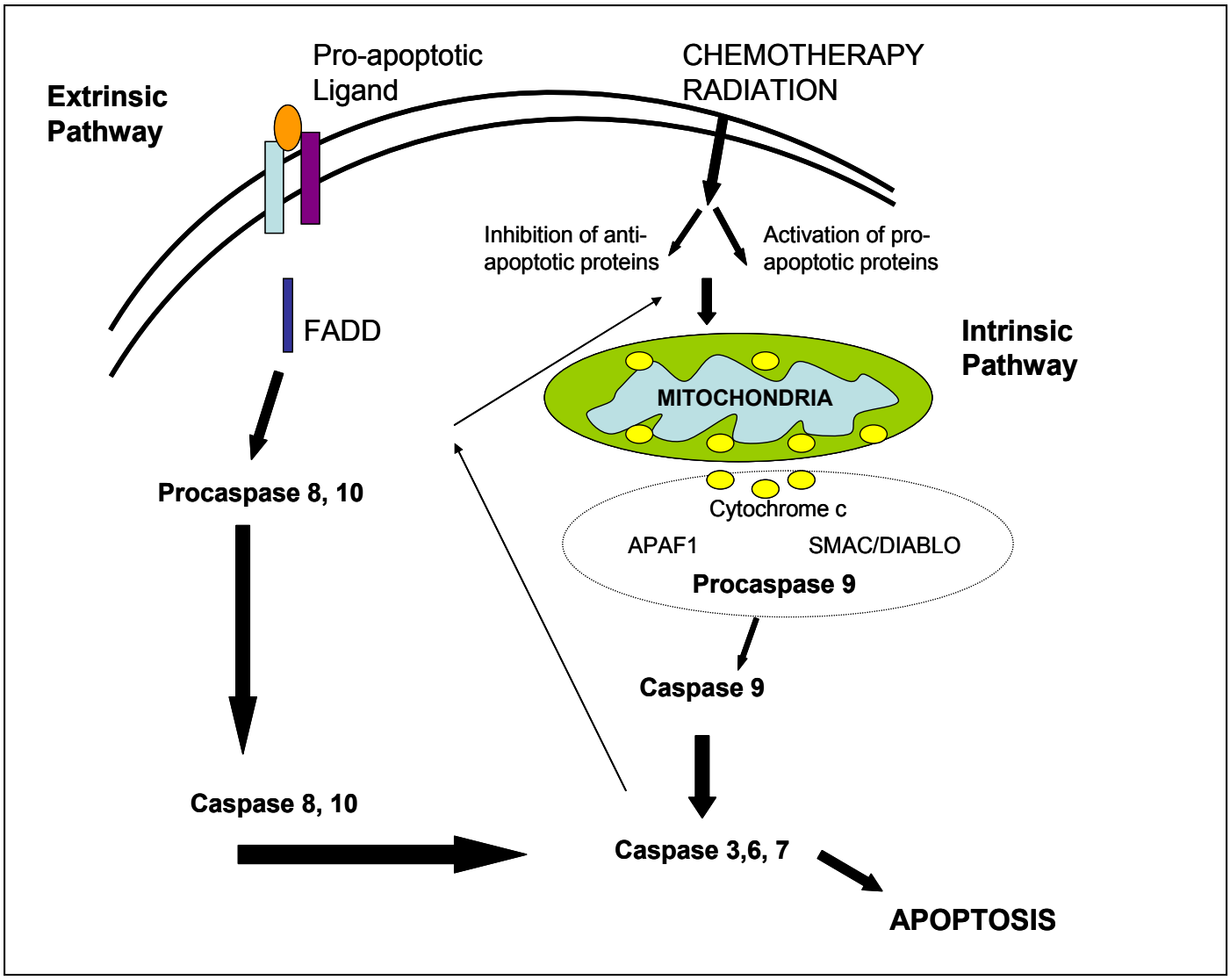

Figure 3 The Intrinsic and Extrinsic Apoptotic Pathways[50].

In the extrinsic pathway, extracellular signals are transduced through activation of receptors in the cellular membrane as specific ligands such as Fas ligand (FasL), tumour necrosis factor α (TNFα), or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) binds to the corresponding death receptor[51](Figure 3). The binding of a ligand to its receptor triggers aggregation of cytosolic receptors and adaptor proteins at the cell membrane[51]. For example, the aggregation of Fas-associated death domain protein (FADD) and initiator caspase 8 creates a death-inducing signal complex known as DISC,

resulting in the activation of caspase 8[51]. Newly activated caspase 8 is able to interact with caspase 3, an effector caspase, and begin the caspase activation cascade[51]. Furthermore, caspase 8 cleaves associated proteins BID and tBID on the mitochondrial membrane and facilitates the release of cytochrome c and initiates the intrinsic apoptotic pathway[51]. Although the initial signal and transduction is uniquely different in each pathway, the pathways converge at the activation of downstream effector caspases. Natural product agents capable of inducing apoptosis through the apoptotic pathways could have an additive effect for the current chemotherapeutic agents which affect cancer cells through other pathways such as cell cycle dysfunction.

1.7 Natural Products and Chemotherapy

There are four major classes of plant-derived anti-cancer drugs: vinca alkaloids, epipodophyllotoxins, taxanes and camptothecin quinolone alkaloid derivatives[1](Figure 4). Vinca alkaloids inhibit cellular proliferation through microtubular dysfunction during mitosis which ultimately leads to apoptosis[1]. The vinca alkaloids specifically induce destabilization through tubulin binding[14]. This binding in turn prevents polymerization[14]. Vinblastine and Vincristine are two examples of Vinca alkaloids. Both are active compounds isolated from the Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don, and are most commonly used in the treatments of leukemias[52]. Researchers have also developed semi-synthetic versions of Vinblastine and Vincristine, which are referred to as Vinorelbine and Vindesine[52]. These semi-synthetic counterparts showed success in the treatment of leukemia, lymphomas, breast cancer and lung cancer, all while exhibiting lower levels of toxicity in animal models[52].

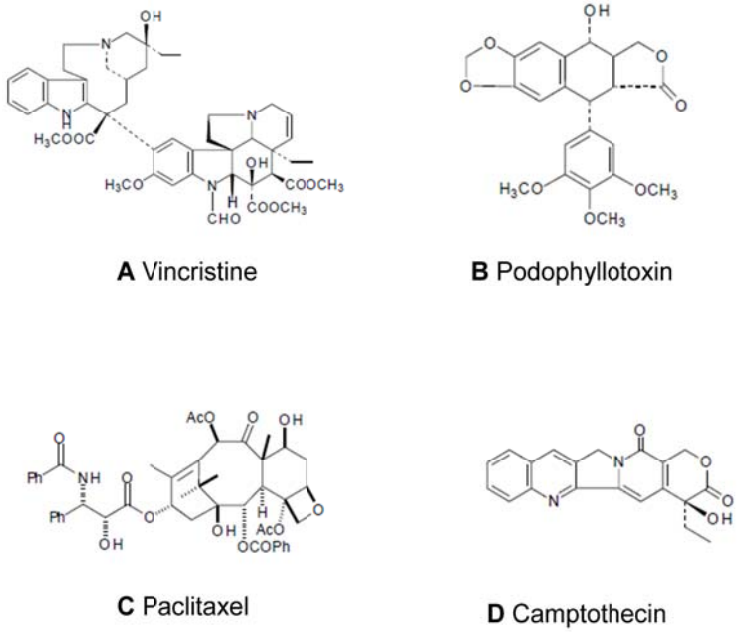

Figure 4 Chemical structures of the four major classes of anti-cancer drugs: A) Vincristine, B) Podophyllotoxin, C) Paclitaxel and D) Camptothecin.

The second class of chemotherapeutic agents are the epipodophyllotoxins. Epipodophyllotoxin is isolated from the roots of *Podophyllum*[53]. Two semi-synthetic analogs that were developed from epipodophyllotoxin are Etoposide and Tenoposide, and are currently used in the treatments of lymphomas, as well as both bronchial and testicular cancers[53]. This class of chemotherapeutic agent inhibits cellular functions of topoisomerase II, an enzyme that is important in the management of DNA tangling and supercoiling[53]. The inhibition of topoisomerase II prevents religation of DNA during replication and consequently blocks progression through the cell cycle[53].

Another group of chemotherapeutic agents belong to the class of Taxanes. Paclitaxel was isolated from the bark of the Pacific Yew[54]. Various deritatives of Paclitaxel have been developed to combat solubility and toxicity issues. Paclitaxel, as well as its derivatives are anti-mitotic drugs[54]. Taxane drugs bind to polymerized microtubules resulting in failed progression through the mitotic phases ultimately causing cell death[52,54]. This class of chemotherapeutic agents is often used in the treatment for patients diagnosed with either breast or ovarian cancer[52,54]. Taxanes function by stabilizing GDP interaction with tubulin in the microtubule and prevents microtubule lengthening or shortening as required to segregate the mitotic spindles during separation of the chromatids during mitosis. This consequently causes cell cycle progression to fail[52,54]. Although the vinca alkaloids and taxanes both affect microtubules function, taxane agents function as mitotic inhibitors, wherease the vinca alkaloids destroy the mitotic spindles[52,54].

The fourth class of drugs are the camptothecin quinoline alkaloid derivatives. Isolated from the bark of *Camptotheca acuminate*, camptothecin, is a cytotoxic alkaloid[52]. Camptothecin inhibits DNA Topoisomerase I functions resulting in replication and transcription dysfunction[52]. Since camptothecin is severely toxic as well as poorly soluble, various analogs were synthesized to optimize the anticancer properties of camptothecin while minimizing the side effects[52]. With so many of the presently used chemotherapeutic agents being derived from a plant source, there is hope that a stronger and more efficient anti-cancer agent has yet to be isolated.

1.8 Proposal

The two most common types of treatments available to cancer patients remain chemotherapy and radiation therapy. These treatments often damage the normal healthy cells within the body resulting in monstrous side effects. Patients often experience increased nausea and fatigue. Cancer patients are also found to describe their state of mind as lethargic while undergoing treatment. The goal of new cancer treatments are shifting to concentrate on minimizing side effects, while increasing the efficiency in treatment targets. Secondly, clinical cancer research is also gradually shifting from reactive responses, to concentrating on cancer preventive measures. It is a combination of these two goals that are promoting the importance of healthy choices through the incorporation of functional foods into the average diet. Our choices today have the potential to influence our future medical profiles, since bioactive food components have the ability to interact with genomic, proteomic, and metabolic factors.

With its increasing popularity, flaxseed has been tested on various diseases including obesity, cardiovascular to even diabetes. For each disease, studies have shown that flaxseed is beneficial in combating the symptoms, as well as reversing the effects of these individual diseases. Unfortunately, the research surrounding flaxseed oil and its' effect on cancer cells has been limited to *in vivo* models and therefore it remains unclear how flaxseed oil, or its' individual components may interact at the cellular level.

We propose to look at the effects of flaxseed oil, the largest plant source of omega 3 fatty acids, on the aggressive murine melanoma cell line B16-BL6 using a series of *in vitro* studies. Furthermore, we propose to look at the metabolic changes in flaxseed oil

following acid metabolism by exposure to B16-BL6 cells using GC/MS analysis. Flaxseed oil has the potential to be a key component of current cancer treatment regimes.

2.0 Material and Methods

2.1 Tissue Culture

The B16-BL6 (murine melanoma), [American Type Culture Collection, Manassas, VA] were maintained in Dulbecco's Modified Essential Medium (DMEM, Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone), 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Burlington, ON). Cells were incubated at 37 ºC in 5% CO_2. For experiments, cells were treated with media containing one of the following plants oils: flaxseed, sunflower, canola, olive, peanut, sesame, and grapeseed. Oils were purchased commercially. Plants oils were mixed directly in media. Low dose treatments consisted of 3 µl of plant oil per ml of media and high dose was 9 µl per ml of media. All experiments were run in triplicate for each assay.

2.2 Gas Chromatography/Mass Spectrometry of Plant Oils

Omega-fatty acid chromatograms were determined for seven plant oils and three brands of flaxseed oil using Gas Chromatography Mass Spectrometry (GC/MS). Analysis of plant oils and the 37 fatty acid methyl ester standards kit (Supelco's standard in CH_2Cl_2) were analyzed on a DB-WAX column (30 m x 0.25 mm x 0.25 um) with a 5 m deactivated fused silica guard column of same dimensions with the following program rate: 50°C, 1 min., 25°C/min. to 200°C, 3°C/min. to 230°C, 8 min. Major peaks were determined by comparing retention times of the plant oil chromatograms with the known retention times of the 37 fatty acid methyl ester standard kit (FAME kit). Table 1 lists the fatty acid composition of each of the analyzed plant oils.

Table 1 Fatty Acid Percentages of the Analyzed Plant Oils

Oil	Alpha Linolenic Acid	Linoleic Acid	Oleic Acid
Flaxseed	57%	16%	18%
Sunflower	4%	65%	16%
Canola	9%	20%	61%
Sesame	5%	41%	44%
Peanut	5%	32%	48%
Grapeseed	< 1%	69%	16%
Olive	< 1%	8%	75%

2.3 Trypan Blue Survival Analysis

Trypan blue survival assay was used to determine the effects of plant oils on cell proliferation in B16-BL6 cells. Cell cultures were prepared at 3 x 10^5 cells/ml confluence on day 0, and B16-BL6 cells were treated with plant oils at low dose (3 µl of plant oil per ml of media) or high dose (9 µl of oil per ml of media), and compared to untreated controls. The plant oil treatments remained in the treated media throughout the length of the experiment. Experiments were 5 days long, and cell counts were recorded for each treatment at 24 hr intervals. Treated B16-BL6 plates were harvested at timed intervals using 0.25% trypsin-EDTA and centrifuged at 1,500 rpm for 10 min. Following harvest, cell pellets were resuspended in PBS, pH 7.4, and incubated with 15 µl of Trypan blue. Stained samples were placed on a hemacytometer and cell counts of clear (live) and blue stained (dead) were recorded for each sample. Experiments were repeated

in triplicate and statistical analysis of cell counts was performed by ANOVA using Graph Pad Prism Software.

2.4 Cell Viability by Methyl Tetrazolium Blue (MTT)

Viability of cells treated with plant oils was determined by a methyl tetrazolium bromide (MTT) reduction assay. Approximately, 3000-5000 cells per well were plated in a 96-well plate and incubated overnight with 5% CO_2 at 37 ºC to allow sufficient time for cells to adhere. B16-BL6 cells were treated with media, or low dose (3 µl/ml media), or high dose (9 µl/ml media) of one plant oil 24hrs following plating. Plant oil treatment remained on cells throughout the length of the 5 day experiment. For solvent experiments, treatments were prepared by mixing plants oils with one solvent (70% Ethanol, DMSO, hexane and dichloromethane) at the previous mentioned concentrations for low and high dose treatments. Solvent preparations were vortexed, incubated for 24 hrs and used to treat B16-BL6 cells. Treatment with solvent only was used as controls. At 24 hr intervals, 10 µl of MTT solution was added to each well of one replicate plate using a multichannel pipettor at a final concentration of 0.5 mg/ml. Plates were incubated at 37 ºC at 5% CO_2 for 3 hrs. 200 µl of dimethyl sulfoxide (DMSO) was added to each well following the incubation period. To ensure full dissolution of purple formazan, cells were repeatedly pipetted. The absorbance was measured at 540 nm using a Titertek Multiskan® MCC/340 plate reader. Experiments were repeated in triplicate and statistical analysis of single concentration points was performed by ANOVA using Graph Pad Prism Software. For recovery experiments, flaxseed oil treated media was removed from wells and replaced with fresh media at timed intervals.

2.5 FACS Analysis: Annexin V and Propidium Iodide Stain

Cell proliferation and cell death were determined by FACS analysis following annexin V and propidium iodide staining. B16-BL6 cells were treated with media, or low dose (3 µl/ml media), or high dose (9 µl/ml media) of one plant oil 24 hrs following plating. Treated B16-BL6 plates were harvested at timed intervals using 0.25% trypsin-EDTA and centrifuged at 1,000 rpm for 10 min. Cells were washed with 1 ml of PBS and transferred to Eppendorf tubes. Cells were centrifuged at 1,000 rpm for 2 mins and supernatant discarded. Cells were blocked in PBS containing 1% FBS for 45 mins. Following blocking, cells were washed with 1 ml PBS, centrifuged at 1,000 rpm for 2 mins and supernatant was removed and discarded. Cells were treated with 5µl of annexin V-FITC stain and incubated for 1 hr. Following incubation, cells were washed with 1 ml PBS, centrifuged at 1,000 rpm for 2 mins and supernatant was removed and discarded. Cells were treated with 5 µl of propidium iodide stain and incubated for 30 mins. Samples were filtered through a syringe to avoid clumping. Experiments were run in triplicate and Annexin V-FITC binding and propidium iodide staining were analyzed by flow cytometry.

2.6 Fluorescence Microscopy: Acridine Orange, Ethidium Bromide, TUNEL, Lysotracker and Mitotracker

Cell death was determined by fluorescence microscopy using the following cellular stains: acridine orange and ethidum bromide, TUNEL, and Lysotracker. Cells were plated at 30% confluence on coverslips in 6-well plates and incubated overnight with 5% CO_2 at 37 ºC to allow sufficient time for cells to adhere. B16-BL6 cells were

treated with media, positive controls (Camptothecin, Cyclohexamide, Rapamycin or UV treated), low dose (3 µl/ml media) plant oil, or high dose (9 µl/ml media) plant oil 24 hrs following plating. Treated B16-BL6 plates were harvested at timed intervals using 0.25% trypsin-EDTA and centrifuged at 1,000 rpm for 10 min. Cells were washed with 1 ml of PBS, fixed in 1ml of 2% paraformaldehyde in PBS and incubated for 5 mins. Cells were washed twice with PBS and incubated with one of the following fluorescence microscopy stains: acridine orange and ethidum bromide, TUNEL, Lysotracker and Mitotracker. Experiments were run in triplicate and cells were photographed by confocal microscope.

2.7 Metabolism Experiment

Omega-3 fatty acid metabolism by B16-BL6 cells was determined by a novel experimental approach. B16-BL6 cells were plated at 30% confluence in large 100 mm tissue culture plates and incubated overnight with 5% CO_2 at 37 ºC to allow sufficient time for cells to adhere. Cells were treated with media, or low dose (3 µl/ml media), or high dose (9 µl/ml media) flaxseed oil 24 hrs following plating. Control media and flaxseed oil treated media were removed from tissue culture plates at 24 hr intervals and used for treatment of a set of five 96-well plates containing B16-BL6 cells which had been previously plated, and would be used in part two of this experiment. This process was repeated at each 24 hr interval and a new set of five 96-well plates was produced for each day of the experiment. In part two of this experiment, 24 hrs following initial treatment of the 96-well plate with the reused media from the initial experiment, one 96-well replicate plate from each set of experimental days was treated with MTT.

Experiments were repeated in triplicate and statistical analysis of single concentration points was performed by ANOVA using Graph Pad Prism Software.

2.8 Gas Chromatography/Mass Spectrometry Analysis of Cellular Membranes

Cells were plated in three 100 mm dishes for each treatment and incubated overnight with 5% CO_2 at 37 ºC to allow sufficient time for cells to adhere. B16-BL6 cells were treated with media, low dose (3 µl/ml media) plant oil, or high dose (9 µl/ml media) plant oil 24 hrs following plating. Cells were lysed with 1.5 ml of methanol (OPTIMA grade, Cat # A454-4, Fisher) with 0.01% BHT (2,6-Di-*tert*-butyl-4-methylphenol, Cat # B1378, Aldrich) using a cell scraper. Cells were collected in a glass tube, sealed and sent to the CCARM laboratory in Winnipeg, Manitoba for GC/MS analysis. The fatty acid composition of B16-BL6 cellular membranes was analysed by gas chromatography (GC) using a Varian 450-GC Gas Chromatograph with an FID detector (Varian, Lake Forest, CA) and a GC capillary column (length 100 m, diameter 0.25 mm and film thickness 0.25 µm; Varian, Lake Forest, CA)[55,56].

3.0 Results

3.1 GC/MS Fame Profiles of Plant Oils

Omega fatty acid profiles of six plant oils and three brands of flaxseed oil were determined by GC/MS. Major omega fatty acids were identified by comparing retention times of the major peaks found in the oil samples to the known retention times (tR) of omega fatty acids from the 37 fatty acid methyl ester standards kit (FAME stds) (Figure 5). Major Peaks found in the plant oils included methyl palmitate (t_R 9.94 min), methyl stearate (t_R 12.02 min), methyl oleate (t_R 12.31 min), methyl linoleate (t_R 12.91 min), and methyl linolenate (t_R 13.84 min). Peanut, canola, sesame and olive oils had a major peak for methyl oleate at 12.31 min retention time. Sesame, grapeseed and sunflower oils had the major peak for methyl linoleate at 12.91 min retention time. Flaxseed oil had the major peak for methyl linolenate at 13.84 min retention time (Figure 5). GC/MS produced similar omega fatty acid profiles for all three brands of flaxseed oil. Although there were some noticeable differences in the sizes of the detected peaks, all three flaxseed oil brands had methyl palmitate (t_R 9.94 min), methyl stereate (t_R 12.02 min), methyl oleate (t_R 12.31 min), methyl linoleate (t_R 12.91 min) and the most significantly detected methyl linolenate (t_R 13.84 min) (Figure 6).

Figure 5 GC/MS Chromatograms of the oils tested and analysis of the FAME (fatty acid methyl esters) standards. A-Fame Stds, B-Peanut, C-Canola, D-Sunflower, E-Flaxseed, F-Sesame, G-Olive and H-Grapeseed.

Analysis of plant oils and the 37 fatty acid methyl ester standards kit (Supelco's standard in CH_2Cl_2) were analyzed on a DB-WAX column (30 m x 0.25 mm x 0.25 um) with a 5m deactivated fused silica guard column of same dimensions with the following program rate: 50°C, 1 min., 25°C/min. to 200°C, 3°C/min. to 230°C, 8 min. Major peaks include: methyl palmitate (t_R 9.94 min), methyl stearate (t_R 12.02 min), methyl oleate (t_R 12.31 min), methyl linoleate (t_R 12.91 min), and methyl linolenate (t_R 13.84 min).

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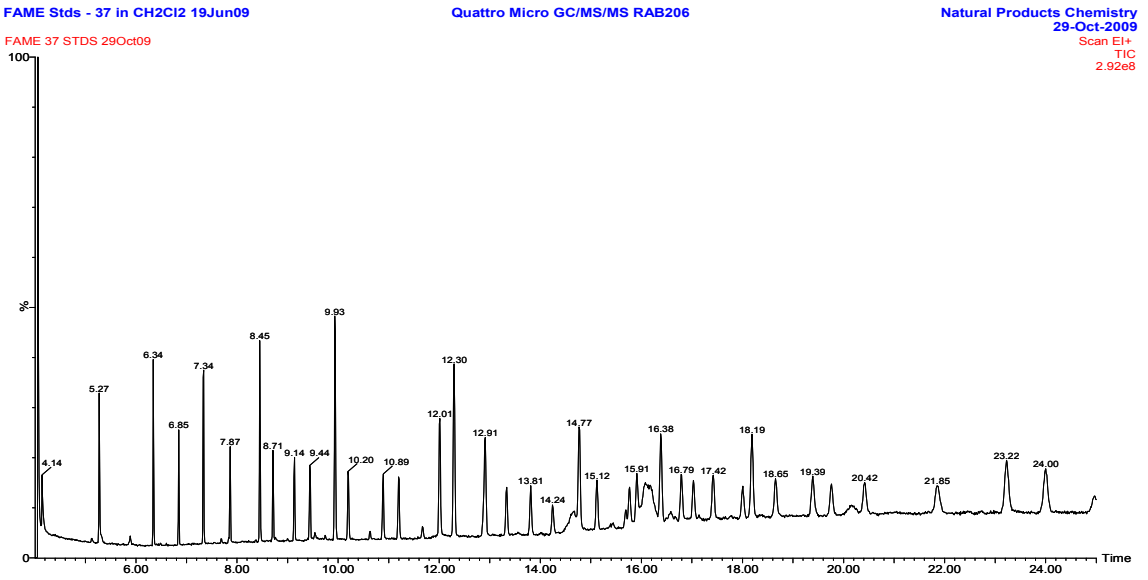
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29-Oct-2009
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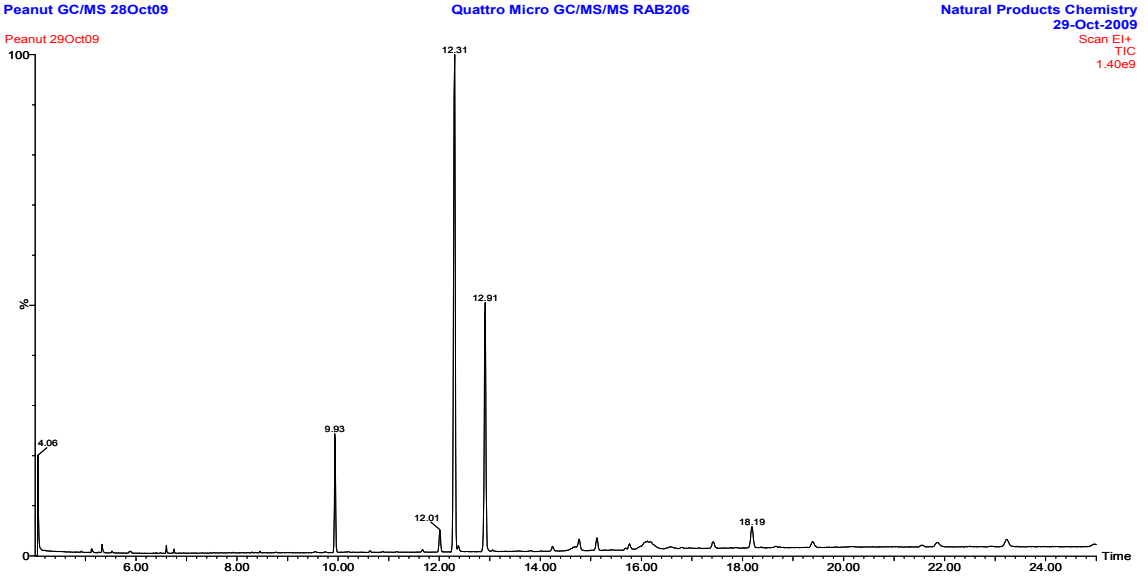
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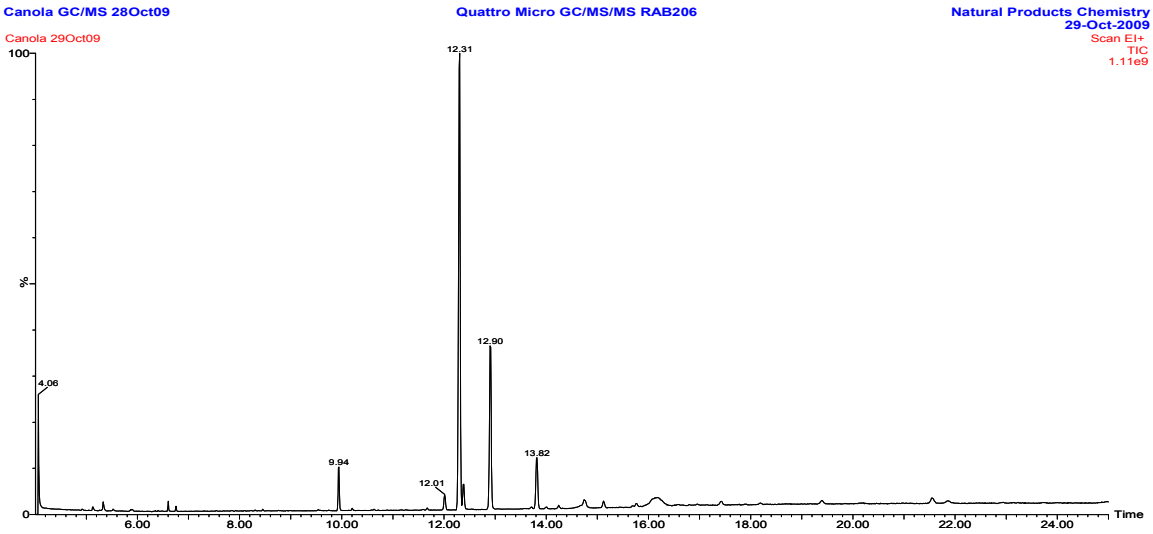
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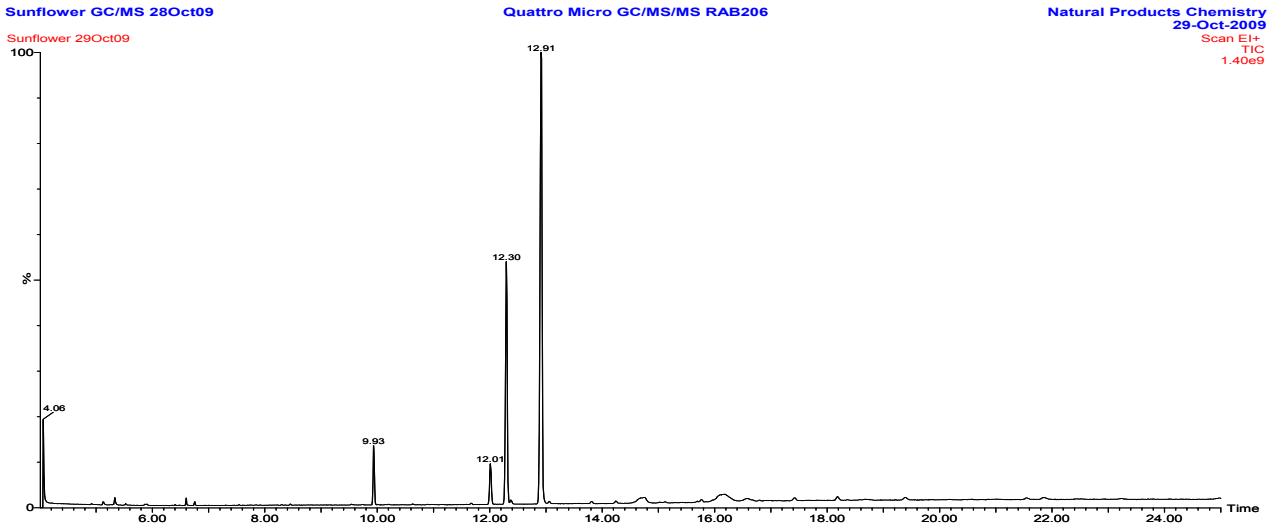
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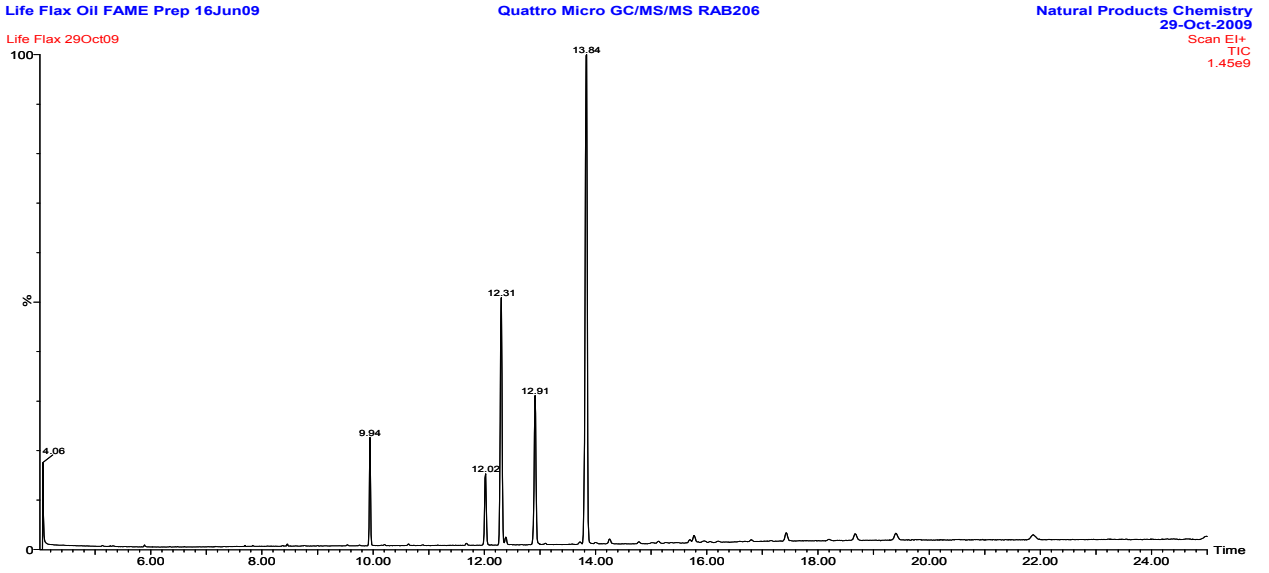
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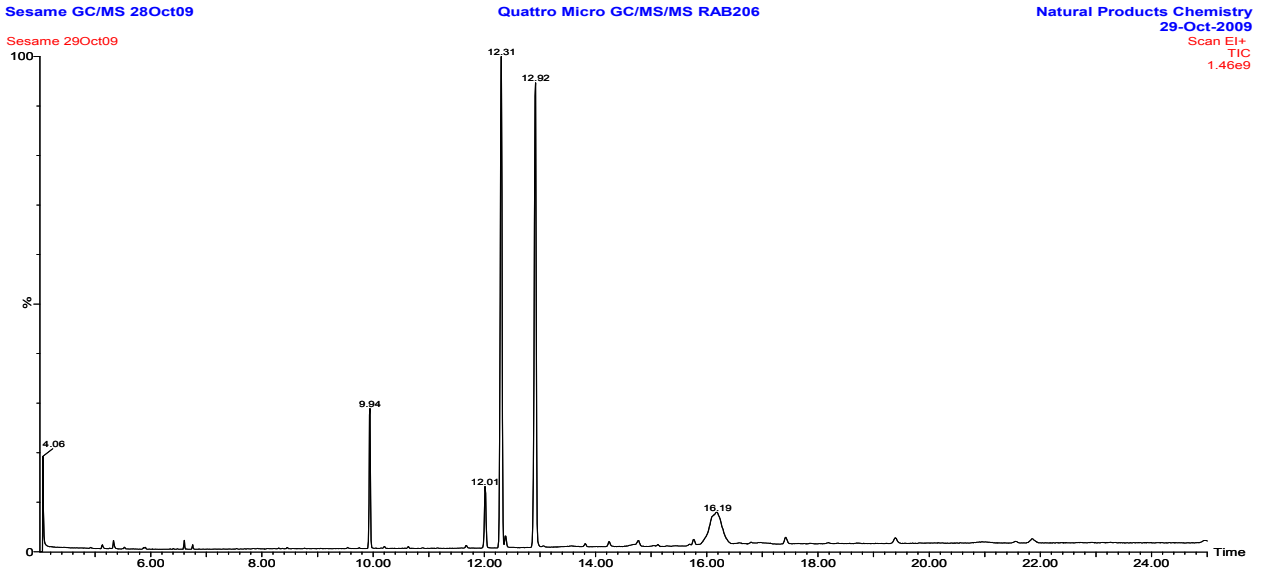
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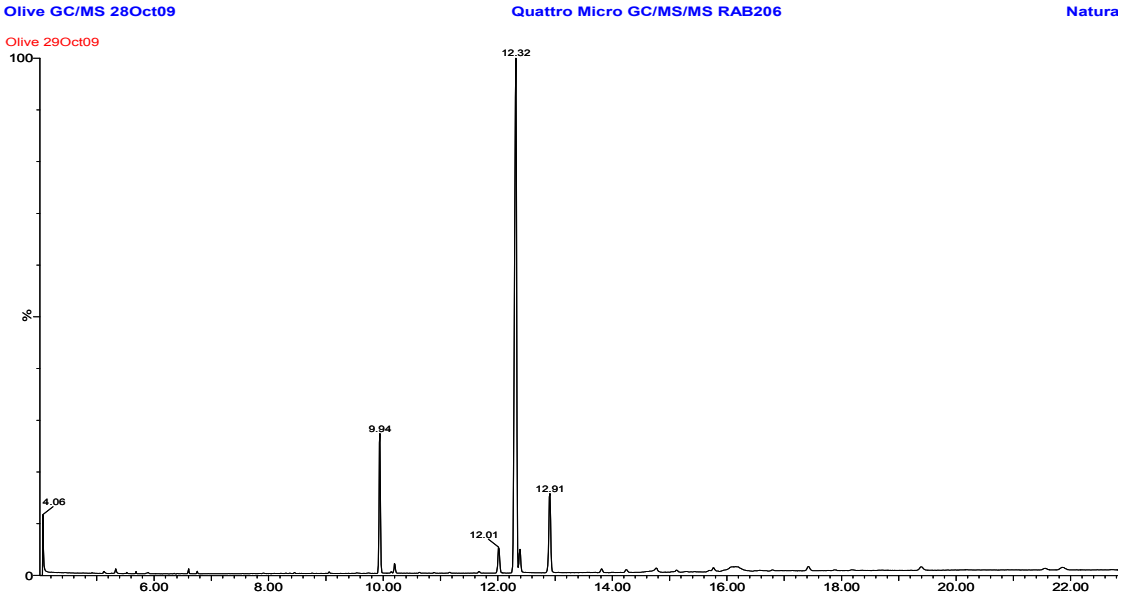
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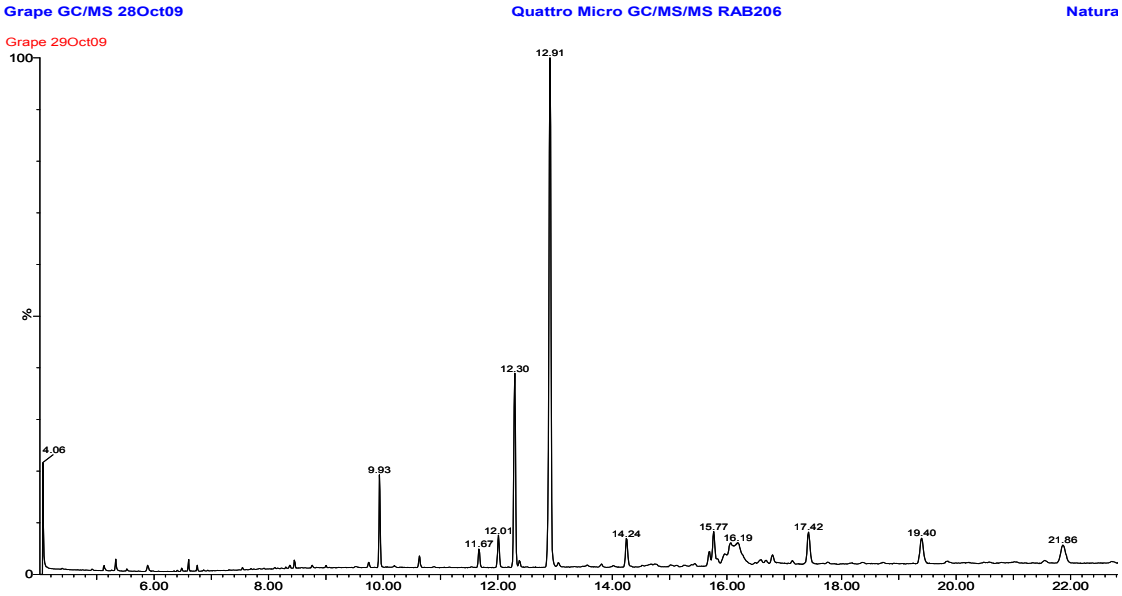
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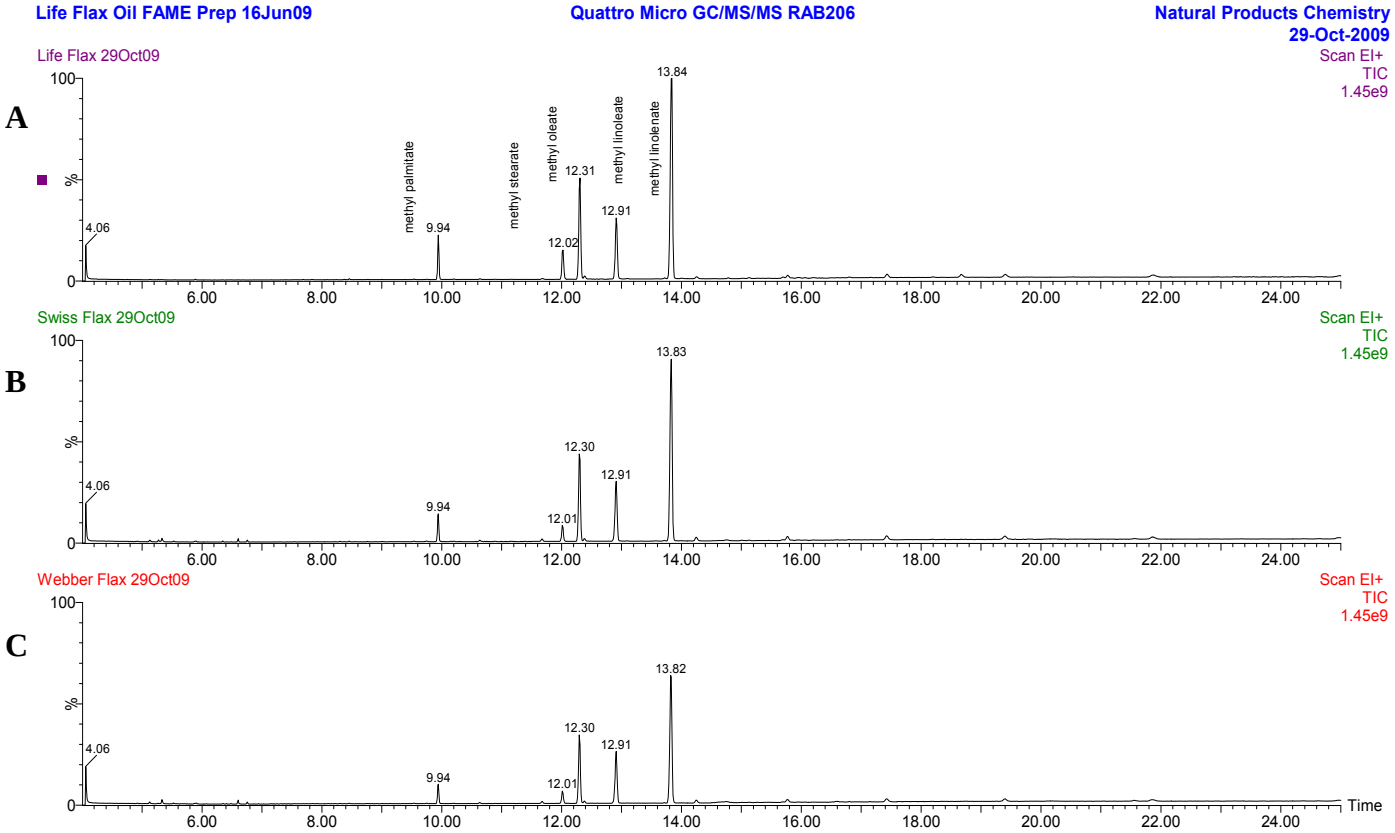
Figure 6 GC/MS Chromatograms of the three brands of flaxseed oil.

Analysis of three brands of flaxseed oil (A- Life Brand, B- Swiss Brand and C-Webber Brand) were analyzed on a DB-WAX column (30 m x 0.25 mm x 0.25 um) with a 5 m deactivated fused silica guard column of same dimensions with the following program rate: 50°C, 1 min., 25°C/min. to 200°C, 3°C/min. to 230°C, 8 min. Major peaks include: methyl palmitate (t_R 9.94 min), methyl stearate (t_R 12.02 min), methyl oleate (t_R 12.31 min), methyl linoleate (t_R 12.91 min), and methyl linolenate (t_R 13.84 min).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29-Oct-2009
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methyl stearate
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methyl linoleate
methyl linolenate
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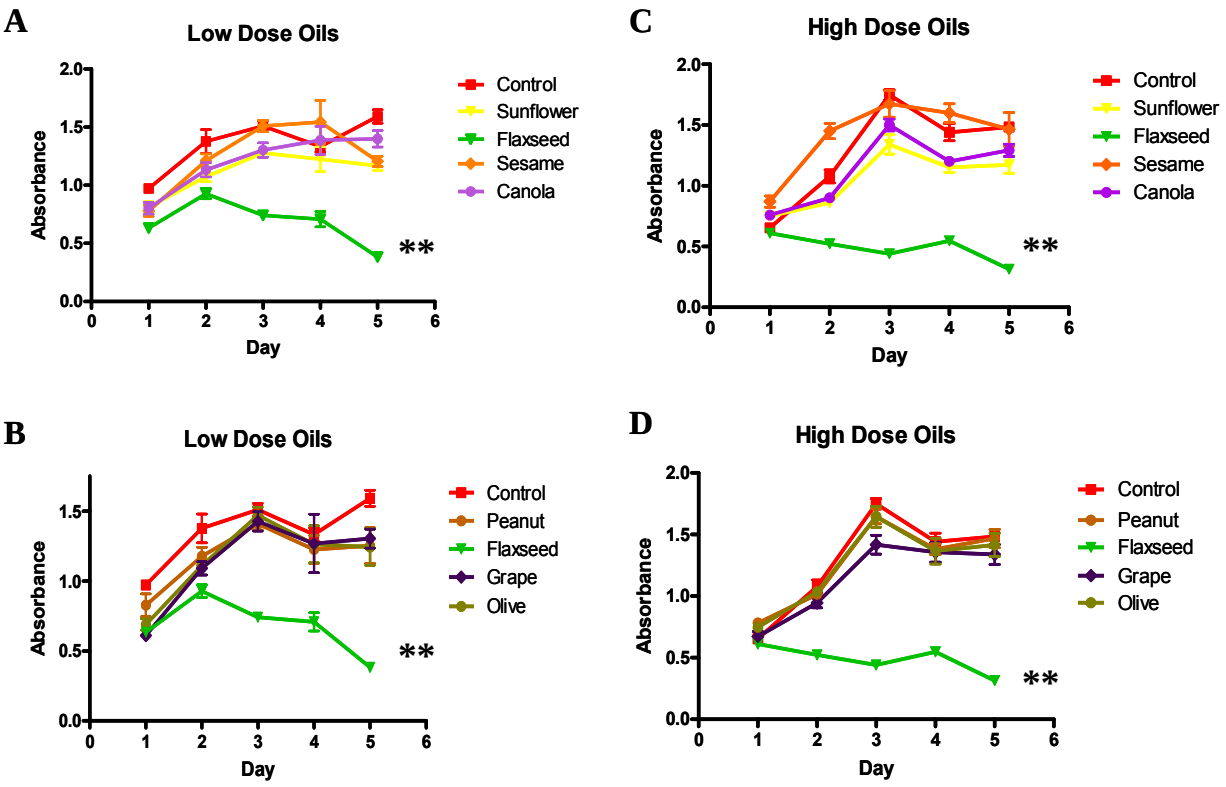
3.2 The effects of plant oils on cell proliferation in B16-BL6 cells

The effect of plant oils on proliferation of the aggressive murine melanoma cell line B16-BL6 was determined using the MTT cell viability assay. A total of seven different oils containing high concentrations of omega fatty acids, including flaxseed oil, olive, sunflower, canola, sesame, peanut and grapeseed, which were previously characterized by HPLC [38] and GC/MS analysis for fatty acid profiles, were used to treat B16-BL6 cells (Figure 5). Although all seven oils contain various amounts of omega-3, -6 or -9 fatty acids, only treatment with flaxseed oil decreased the growth of the aggressive murine melanoma cell line B16-BL6 ($p < 0.05$) (Figure 7) . Treatment of B16-BL6 with each of the other six characterized oils showed no significant change in cell growth starting at 48 hrs post treatment ($p< 0.001$) (Figure 7). Furthermore, this decrease in cell growth in response to flaxseed oil treatment was dose dependent. Treatment with high dose flaxseed oil (9 µl/ml of media) had significantly more cell growth inhibition when compared to low dose flaxseed oil (3 µl/ml of media). Significant changes in cell growth were detected at day 3 for low dose flaxseed oil treatment and as early as day 2 for high dose flaxseed oil treatment. For both doses, cell viability continuously decreased over the length of the experiment.

Figure 7 The effect of plant oils on cell viability of the aggressive murine melanoma B16-BL6 using MTT assays

B16-BL6 cells treated with either low dose (3 µl/ml) of plant oils (A-sunflower, flaxseed, sesame, canola B-peanut, grape and olive) or high dose (9 µl/ml) of plant oils (C-sunflower, flaxseed, sesame, canola B-peanut, grape and olive) in culture media starting on day 0. Control represents treatment with culture media only. The cells were treated with the indicated oils at low dose (3µl/ml) and high dose (9µl/ml) on day 0 and maintained for the duration of the experiment. Each day of treatment, for 5 days, the number of cells in each well of the plate was determined using the MTT assay.

(** = $p < 0.01$)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Low Dose Oils
Absorbance
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Control
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Canola
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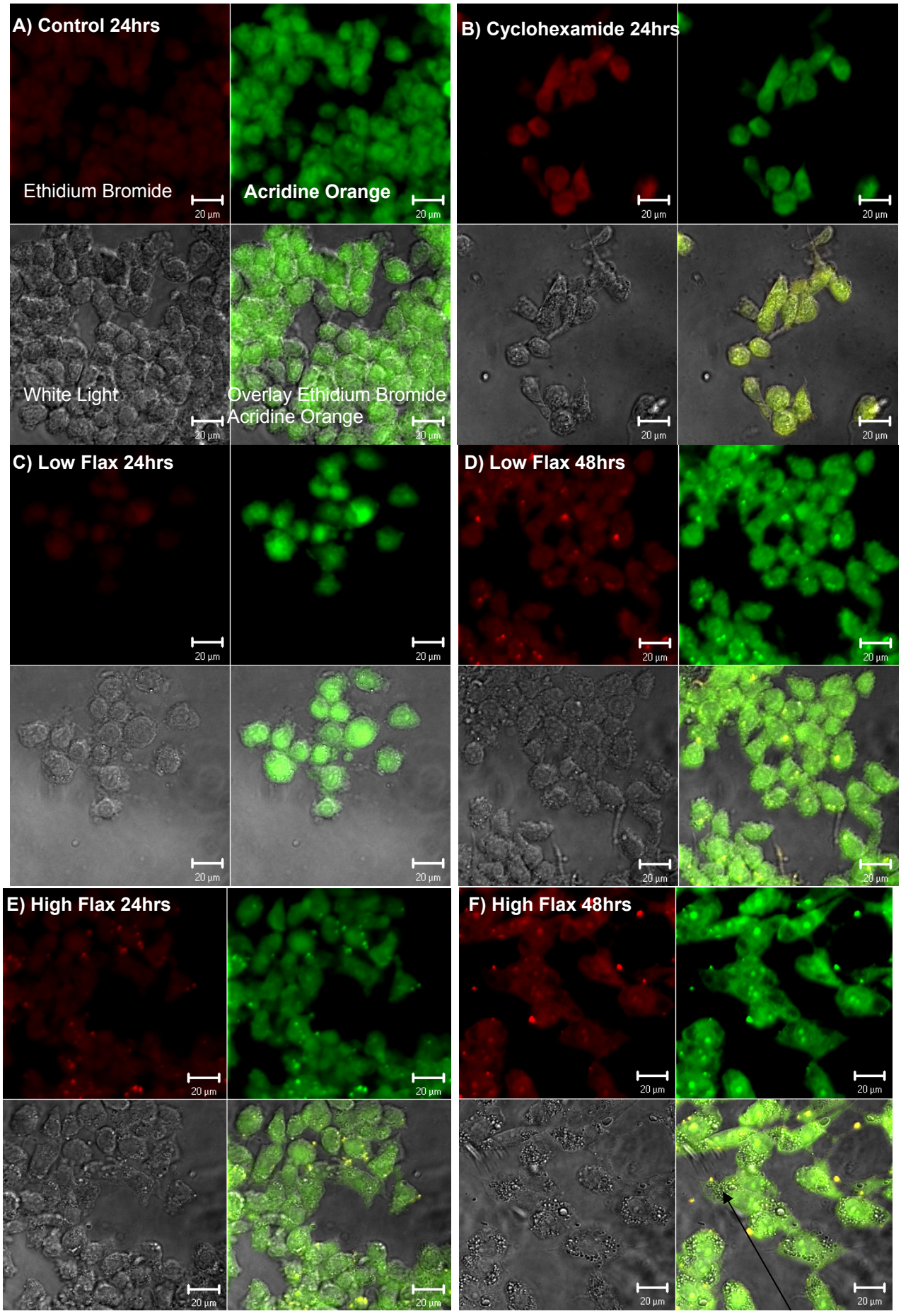
Absorbance
Day
Control
Peanut
Flaxseed
Grape
Olive
**
C
High Dose Oils
Absorbance
Day
Control
Sunflower
Flaxseed
Sesame
Canola
**
D
High Dose Oils
Absorbance
Day
Control
Peanut
Flaxseed
Grape
Olive
**

3.3 Flaxseed oil treatment induces apoptosis in B16-BL6 cells

The induction of apoptosis was determined by investigating cellular morphological changes on the confocal microscope using acridine-orange and ethidium bromide (Figure 8). Cells were plated onto coverslips in 6-well tissue culture plates. Following a 24 hr incubation period, cells were treated with either low dose (3 μl/ml of media) or high dose (9 μl/ml of media) flaxseed oil and compared to untreated controls or the positive control cyclohexamide. The untreated controls had background levels of acridine orange stain present and minimal ethidium bromide staining. These results are indicative of viable B16-BL6 cells. The positive control cyclohexamide had increased levels of ethidium bromide staining at 24 hrs, suggesting changes in cell viability and potentially increased apoptosis, a type of cell death often described as cell suicide. Low flaxseed oil treatment at 24 hrs had minimal detection of ethidium bromide. This finding suggests that 24 hrs may be too short of a time period for cells to undergo apoptosis following exposure to low doses of flaxseed oil treatment. However, at 48 hrs there was significant increases in ethidium bromide stain in the low dose flaxseed oil treated cells. Furthermore, at 48 hrs apoptotic bodies could also be detected within the cells. These apoptotic bodies can be visualized in the high dose flaxseed oil treatments as early as 24 hrs following treatment. At 48 hrs, cells treated with flaxseed oil had significant amounts of apoptotic bodies as well as high detection of ethidium bromide in the cells. For all four flaxseed oil conditions, changes in cellular morphology including membrane blebbing can be visualized in the white light microscopy pictures (Figure 8).

Figure 8 Apoptosis analysis by acridine orange and ethidium bromide staining of flaxseed oil treated B16-BL6 cells

B16-BL6 cells were grown on slide coverslips and treated with either low dose (3 µl/ml) or high dose (9 µl/ml) flaxseed oil treatments over 48 hrs, and compared to the negative untreated controls and the positive cyclohexamide controls. (A-24 hr untreated negative controls, B-6 hr positive cyclohexamide treated controls, C-24 hr low dose flaxseed oil, D-48 hr low dose flaxseed oil, E-24 hr high dose flaxseed oil and F-48 hr high dose flaxseed oil. Significant morphological changes such as membrane blebbing are visible in all flaxseed oil treated conditions (C,D,E and F). Apoptotic bodies are present in low dose 48 hr flaxseed oil treated cells (D) and in high dose 24 hrs (E) and 48 hrs (F) flaxseed oil treated cells.


A) Control 24hrs
Ethidium Bromide
Acridine Orange
White Light
Overlay Ethidium Bromide
Acridine Orange
B) Cyclohexamide 24hrs
C) Low Flax 24hrs
D) Low Flax 48hrs
E) High Flax 24hrs
F) High Flax 48hrs
20 µm
Apoptotic Bodies

Apoptosis and DNA damage following flaxseed oil treatment was further investigated using TUNEL, a stain that utilizes terminal deoxynucleotidyl transferase (TdT) to label dUTP nick ends (Figure 9). B16-BL6 cells were cultured onto coverslips and treated with low dose or high dose flaxseed oil or sunflower oil treatments. These treated cells were then compared to the negative untreated controls and the positive camptothecin treated control. The untreated control and all conditions of sunflower oil treated cells showed no signs of TUNEL stain above the background level detections (Figure 9). These findings indicate that untreated controls as well as the sunflower treated cells do not have significant DNA damage and therefore are not undergoing apoptosis. At 24 hrs, the positive control camptothecin did have slightly increased TUNEL staining, a positive marker for DNA strand breaks and early apoptosis. Positive brightly stained points were detected in every flaxseed oil condition to some degree. The TUNEL stain was dose-dependent as well as time dependent for the flaxseed oil treatments, with the brightest stains in respect to DNA damage detected in the high flaxseed oil dose at 48 hrs (Figure 9). This finding supports the idea that the omega-3 rich flaxseed oil is capable of inducing apoptosis in B16-BL6 cells, while the omega-6 rich sunflower oil is not (Figure 9). Furthermore, annexin-V-FITC was used to determine the percentage of cell death in B16-BL6 cells following flaxseed oil treatment (Figure 10). Cells were treated with low dose or high dose of flaxseed oil or sunflower oil and compared to untreated negative controls and positive controls following 24 hrs and 48 hrs time periods. Cells treated with low dose flaxseed oil had significantly higher detection of annexin V-FITC antibody at 48 hrs with 32% of its cell population detected for positive stain and 53% of its cell population stained positive at 72 hrs (Figure 10-C). The

untreated negative controls had approximately 14% of its cell population stain positive for annexin V-FITC at 24 hrs, 48 hrs and 72 hrs time points (Figure 10-B). The cells treated with high dose flaxseed oil had the largest populations of positive stained annexin V-FITC at 52% and 45% for the 24 hr and 48 hr time points, respectively (Figure 10-D). The cells treated with high dose flaxseed oil at 72 hrs had fully compromised cellular membranes that the entire cell population was detected as positively stained at 98% (Figure 10-D). These findings further support our previous results that flaxseed oil at low dose and as early as 24 hrs is capable of inducing apoptosis in B16-BL6 cells. Sunflower oil treated cells did not show significant increases in annexin V-FITC detection at both low dose and high dose treatments for all three time points (Figure 10-E and F). This finding is indicative of viable of cells and supports our previous findings that sunflower oil does not induce apoptosis in B16-BL6 cells.

Figure 9 Early stage apoptosis is detected by TUNEL stain in B16-BL6 cells following flaxseed oil treatment.

B16-BL6 cells grown on coverslips were exposed to either low dose (3 μl/ml) or high dose (9 μl/ml) of flaxseed oil or low or high dose sunflower oil in culture media starting on day 0. The negative control represents treatment with only culture media, while the positive controls are cells exposed to 10^{-6} M camptothecin. Cells were cultured in the presence of the indicated agents for 24 hr or 48 hr and then fixed and stained with TUNEL staining kit following the manufacturer's instructions.

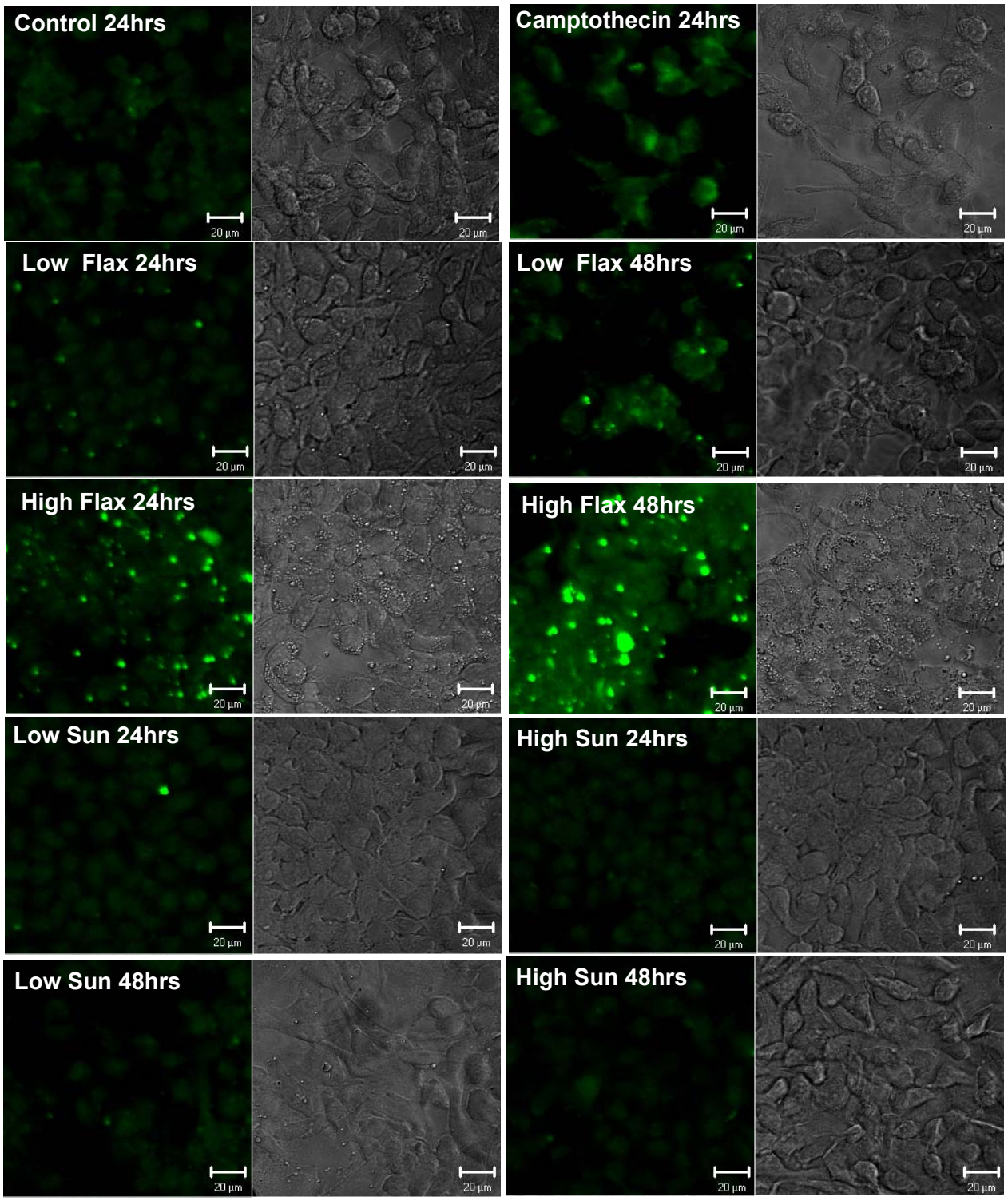
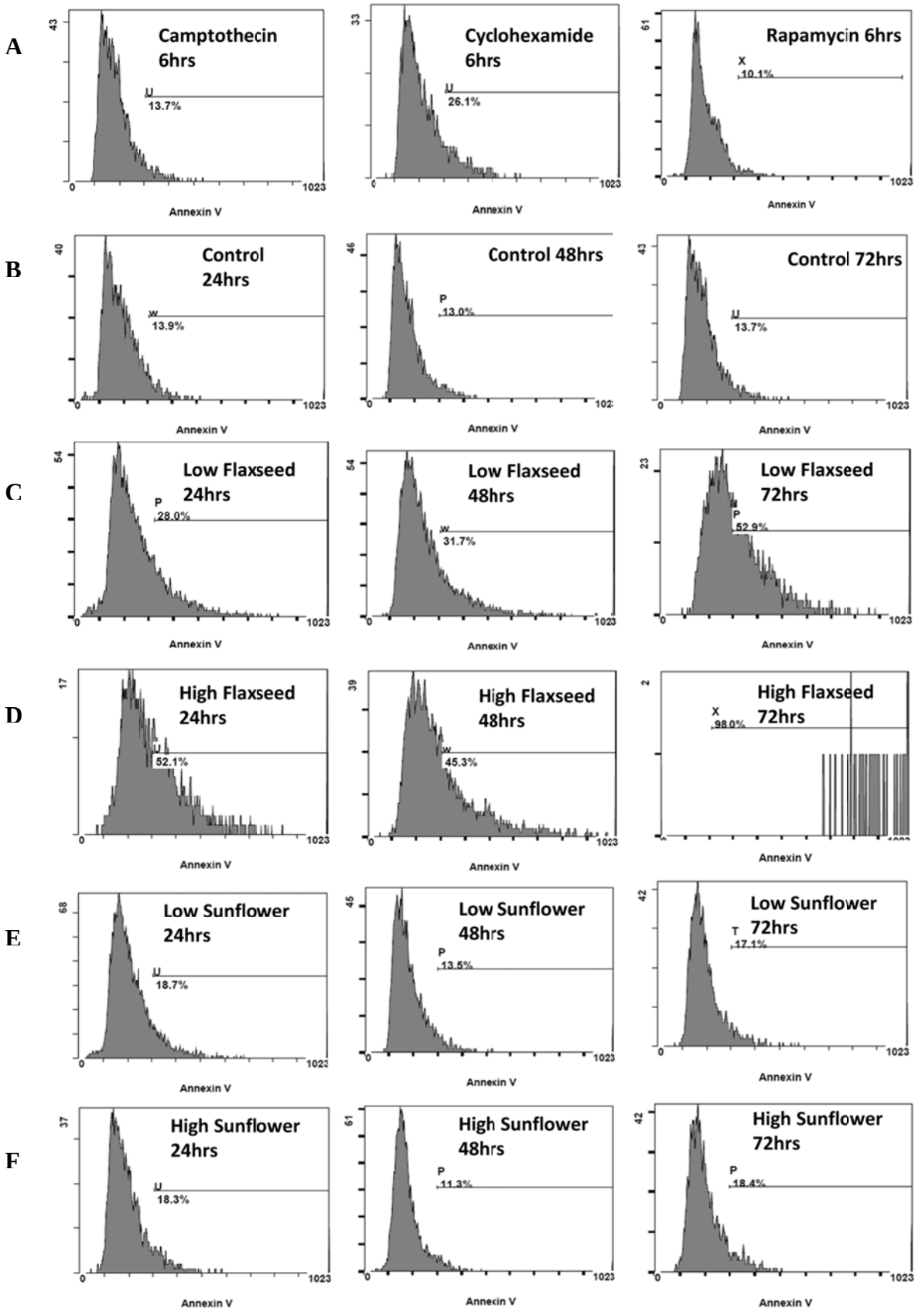

Control 24hrs
Camptothecin 24hrs
Low Flax 24hrs
Low Flax 48hrs
High Flax 24hrs
High Flax 48hrs
Low Sun 24hrs
High Sun 24hrs
Low Sun 48hrs
High Sun 48hrs
20 µm

Figure 10 Cell death in B16-BL6 cells determined by annexin V-FITC staining following flaxseed oil treatment.

B16-BL6 cells grown in medium tissue plates and exposed to, either low dose (3 μl/ml) or high dose (9 μl/ml) of flaxseed oil or high or low dose of sunflower oil in culture media starting on day 0. The negative control represents treatment with only culture media, while the positive controls are cells exposed to camptothecin, rapamycin, cyclohexamide or 15 mins of exposure to ultraviolet light. Harvested, fixed with 70% EtOH at -20ºC, blocked and stained by incubating with annexin V-FITC. The % cells were determined as those with greater fluorescence than unstained control cells. (A-positive treated controls camptothecin 6 hrs, cyclohexamide 6 hrs and rapamycin 6 hrs, B-untreated negative controls 24 hrs, 48 hrs and 72 hrs, C-low dose flaxseed oil 24 hrs, 48 hrs and 72 hrs, D-high dose flaxseed oil 24 hrs, 48 hrs and 72 hrs, E-low dose sunflower oil 24 hrs, 48 hrs and 72 hrs, F-high dose sunflower oil 24 hrs, 48 hrs and 72 hrs)

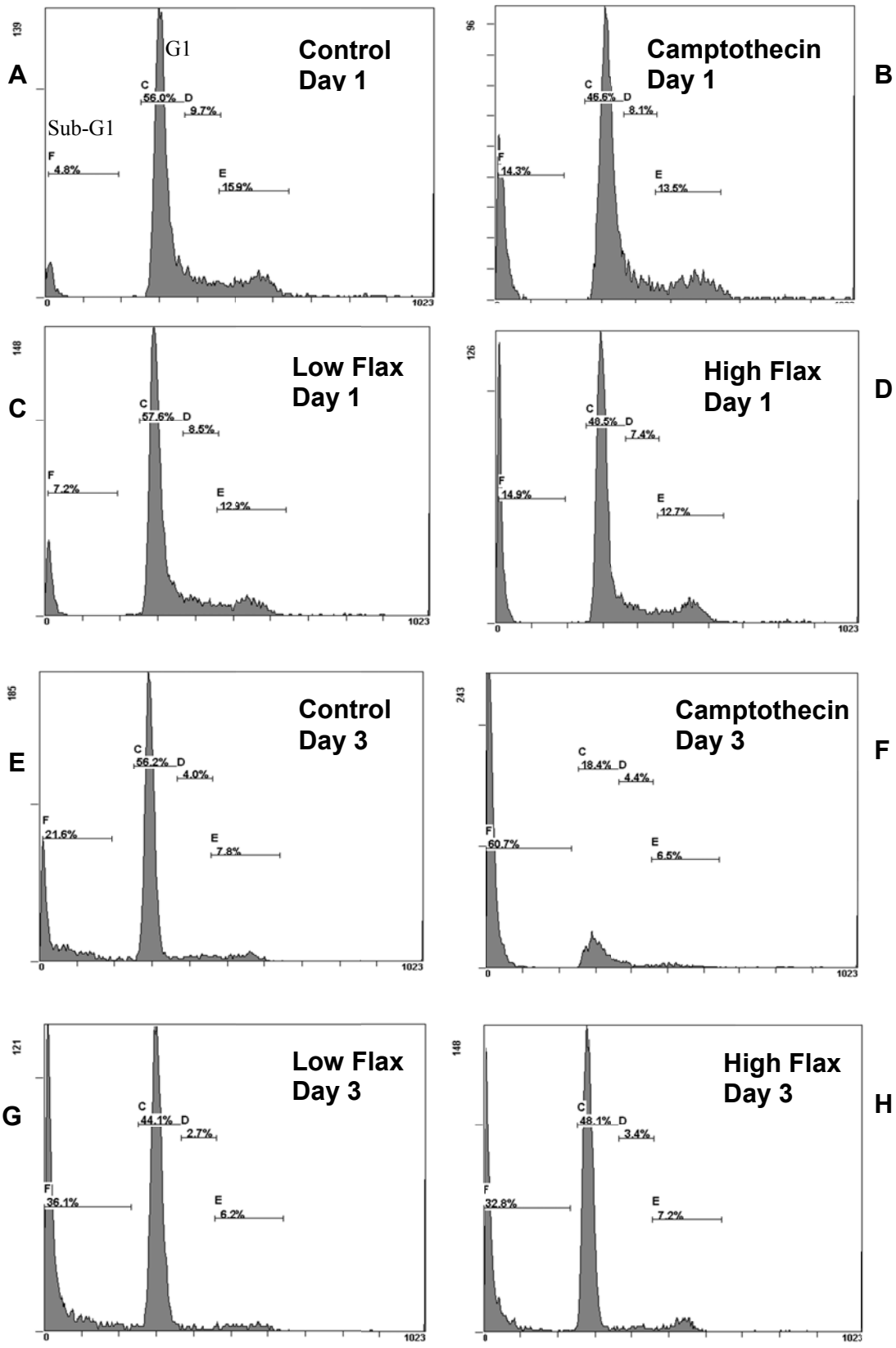

A
Camptothecin 6hrs
13.7%
Cyclohexamide 6hrs
26.1%
Rapamycin 6hrs
10.1%
B
Control 24hrs
13.9%
Control 48hrs
13.0%
Control 72hrs
13.7%
C
Low Flaxseed 24hrs
28.0%
Low Flaxseed 48hrs
31.7%
Low Flaxseed 72hrs
52.9%
D
High Flaxseed 24hrs
52.1%
High Flaxseed 48hrs
45.3%
High Flaxseed 72hrs
98.0%
E
Low Sunflower 24hrs
18.7%
Low Sunflower 48hrs
13.5%
Low Sunflower 72hrs
17.1%
F
High Sunflower 24hrs
18.3%
High Sunflower 48hrs
11.3%
High Sunflower 72hrs
18.4%
Annexin V

3.4 Cell Cycle and Inhibition of Cell Proliferation

Cell cycle changes following flaxseed oil treatment was investigated by propidium iodide detection by flow cytometry. Changes in phases of cell cycle in cell populations can be determined by the quantity of genetic material. Following 24 hrs, plated cells were treated with either low dose or high dose flaxseed oil and compared to the negative untreated controls, and the positive camptothecin treated controls (Figure 11). The untreated controls had the majority of their cell population (56%) detected in the in the G1 phase and approximately 5% and 22% of cells in sub-G1 at day 1 and day 3, respectively (Figure 11- A and E). The positive control camptothecin had increased cell populations in the sub-G1 phase. Cells treated with camptothecin had 14% of its populations in sub-G1 phase on day 1 and over 60% of its cell populations in sub-G1 on day 3 (Figure 11- B and F). Low dose flaxseed oil treated cells had 58% and 44% of cells in G1, and 7% and 36% of cells in sub-G1 on days 1 and 3, respectively (Figure 11- C and G). High dose flaxseed oil treatment had 48% of its cells in G1 phase for both days 1 and 3. Approximately 15% and 33% of cells were detected in sub-G1 for day 1 and day 3, respectively (Figure 11- D and H). These results suggest that both low dose and high dose flaxseed oil are effective in significantly increasing cell death in B16-BL6 cells by day 3 (Figure 11- G and H).

Figure 11 Cell cycle analysis of B16-BL6 cells treated with flaxseed oil.

Cell cycle analysis of B16-BL6 cells was determined using propidium iodide stain and flow cytometry. Cells were treated with high dose (9 µl/ml), low dose (3 µl/ml), or culture media on day 0 and maintained for the duration of the experiment. Camptothecin was used as a positive control. (A-untreated negative control day 1, B-positive camptothecin control day 1, C-low dose flaxseed oil day 1, D-high dose flaxseed oil day 1, E-untreated negative control day 3, F-positive camptothecin control day 3, G-low dose flaxseed oil day 3 and H-high dose flaxseed oil day 3). All experiments were run in triplicate and data were analyzed for 3 experiments.

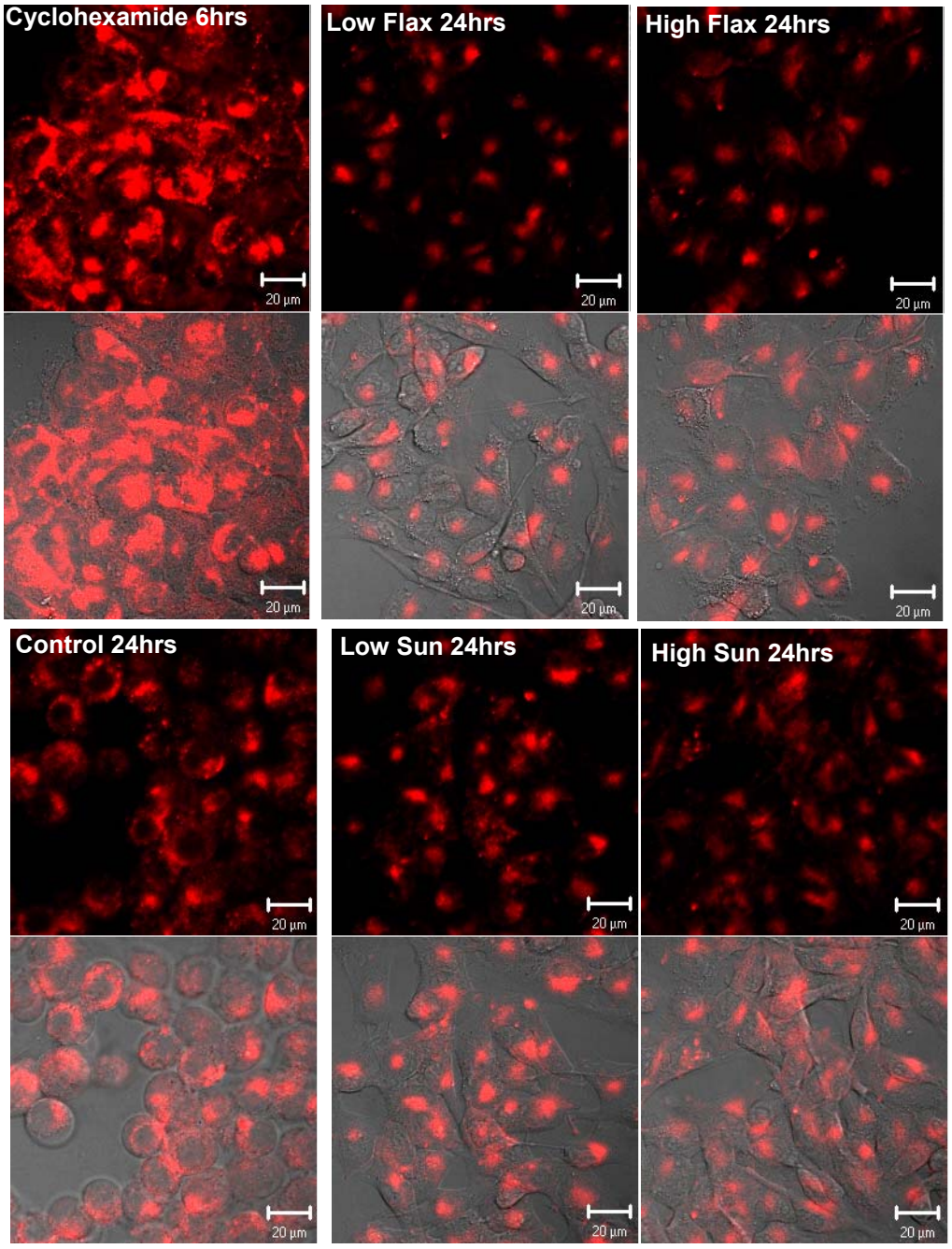

A
Control
Day 1
G1
Sub-G1
C
56.0%
D
9.7%
F
4.8%
E
15.9%
B
Camptothecin
Day 1
C
46.6%
D
8.1%
F
14.3%
E
13.5%
C
Low Flax
Day 1
C
57.6%
D
8.5%
F
7.2%
E
12.3%
D
High Flax
Day 1
C
49.5%
D
7.4%
F
14.9%
E
12.7%
E
Control
Day 3
C
56.2%
D
4.0%
F
21.6%
E
7.8%
F
Camptothecin
Day 3
C
18.4%
D
4.4%
F
60.7%
E
6.5%
G
Low Flax
Day 3
C
44.1%
D
2.7%
F
36.1%
E
6.2%
H
High Flax
Day 3
C
48.1%
D
3.4%
F
32.8%
E
7.2%

3.5 Lysosome formation in flaxseed oil treated B16-BL6 cells

Lysosome formation in B16-BL6 cells following flaxseed oil treatment was investigated using Lysotracker, a fluorescent probe capable of labeling acidic organelles. It was unclear whether the uptake and metabolism of the added fatty acids involved phagocytosis and lysosomal degradation. Cells plated on coverslips were treated with either low dose or high dose of flaxseed oil, sunflower oil, or untreated media culture (Figure 12). The positive control cyclohexamide showed significant increases in lysotracker stain. All other conditions, including untreated controls, flaxseed oil and sunflower oil treated conditions showed no significant changes in lysotracker stain (Figure 12). This result suggests that neither flaxseed oil nor sunflower oil is capable of influencing lysosome presence in B16-BL6 cells.

Figure 12 Lysosome formation analysis in flaxseed oil treated B16-BL6 cells by lysotracker stain

The formation of lysosomes following flaxseed oil treatment was determined in B16-BL6 cells. Cells were treated with high dose (9 µl/ml), low dose (3 µl/ml), of either flaxseed oil or sunflower oil and compared to untreated negative controls. Cyclohexamide was used as a positive control.


Cyclohexamide 6hrs
Low Flax 24hrs
High Flax 24hrs
Control 24hrs
Low Sun 24hrs
High Sun 24hrs
20 µm

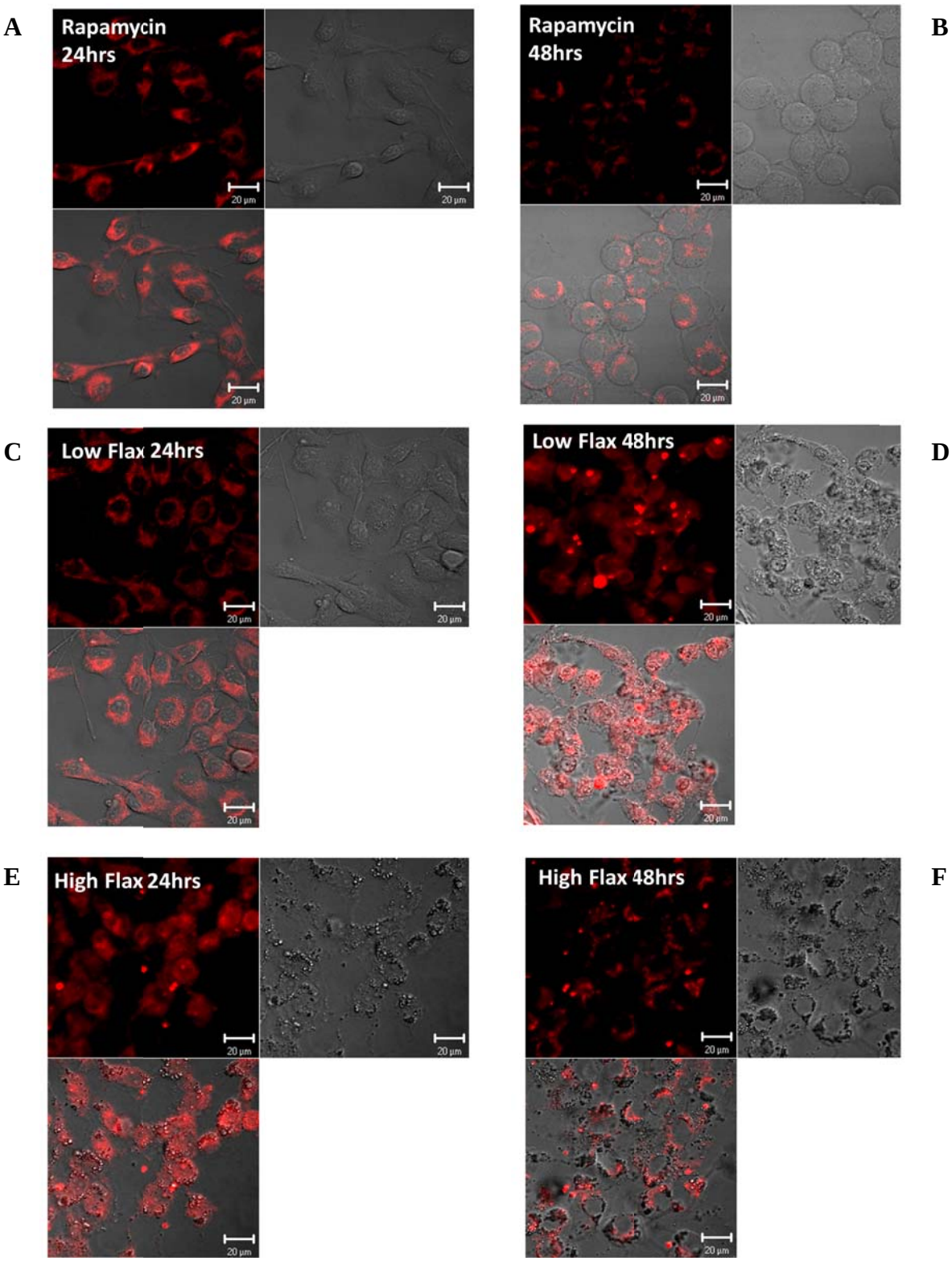
3.6 Mitochondrial Changes in Flaxseed Oil Treated B16-BL6 cells

Mitotracker red is a fluorescent red dye used to stain mitochondria in cells. Since the accumulation of mitotracker red within the cell is dependent on membrane potential, this stain is often used in determining apoptosis following treatments. Cells were plated on coverslips and incubated overnight at 37°C. Cells were treated with low or high dose of flaxseed oil or sunflower oil. Rapamycin treatment at 6 mM was used as a positive control, while untreated cells were used as negative controls. Cells were left in treatment for 24 hr and 48 hr time periods. At collection, live cells were washed with PBS, stained with mitotracker and then fixed using paraformaldehyde. Once coverslips were mounted on slides, cells were observed using a confocal microscope. The untreated negative controls as well as the sunflower oil treated cells showed large amounts of mitotracker red staining within the cells (Figure 13-G and H). Furthermore, the stain was perinuclear and granulated. This type of staining is indicative of many healthy viable mitochondria within the cells (Figure 13-G and H). The sunflower oil treated cells at both low dose and high dose, as well as at both 24 hr and 48 hr time points resembled the healthy viable cells detected in the untreated negative controls (Figure 13-I and J). The sunflower oil treated cells had large amounts of staining that was primarily perinuclear and granulated (Figure 13-I and J). The positive control rapamycin treated cells had less staining at 24 hrs and minimal staining at 48 hrs (Figure 13-A and B). These findings suggest compromised mitochondria and apoptotic cells. The flaxseed oil treated cells showed small amounts of detectable mitotracker stain in their cells (Figure 13-C, D, E and F). Perinuclear staining was detectable in the low flaxseed oil treatment at 24 hrs (Figure 13-C). This perinuclear staining could not be detected in cells treated with low flaxseed oil

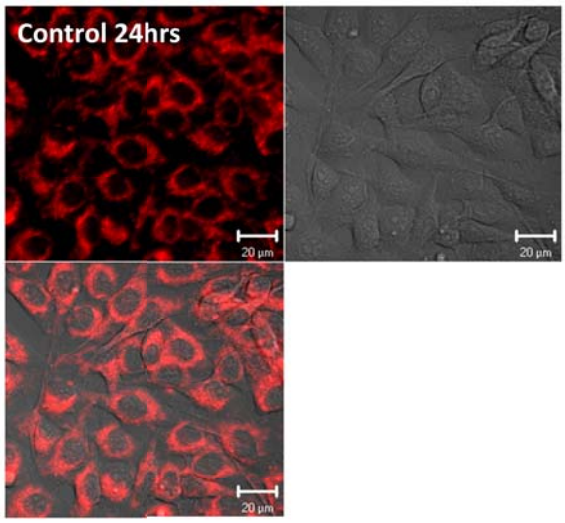
for 48 hrs, and for both high flaxseed oil treatments at 24 hrs and 48 hrs (Figure 13-D, E and F). These treated cells had minimal background mitotracker red detection and very bright saturated points of staining within the cells. These findings suggest that mitochondrial activity is greatly affected by flaxseed oil treatments at low doses and at timepoints as early as 24 hrs (Figure 13-D, E and F). Furthermore, the loss of viable mitochondrial is a characteristic associated with apoptosis.

Figure 13 Mitochondrial analysis of flaxseed oil treated B16-BL6 cells using mitotracker red

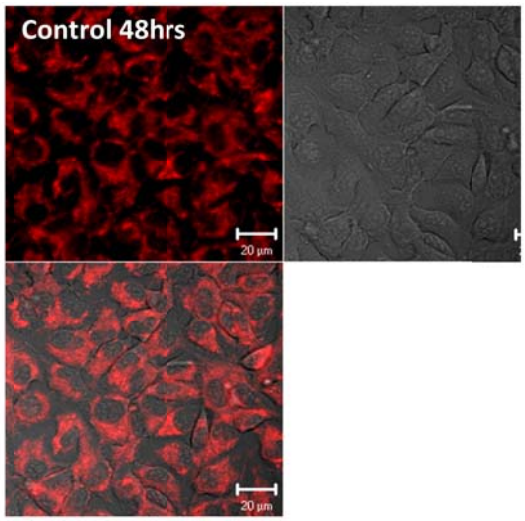
The effect of flaxseed oil treatment on the viability of mitochondria was determined in B16-BL6 cells. Cells were treated with high dose (9 µl/ml), low dose (3 µl/ml), of either flaxseed oil or sunflower oil and compared to untreated negative controls. Rapamycin was used as a positive control. (A-positive rapamycin control 24 hrs, B-positive rapamycin control 48 hrs, C-low dose flaxseed oil 24 hrs, D-low dose flaxseed oil 48 hrs, E-high dose flaxseed oil 24 hrs, F-high dose flaxseed oil 48 hrs, G-untreated negative controls 24 hrs, H-untreated negative controls 48 hrs, I-low dose sunflower oil 24 hrs, J-low dose sunflower oil 48 hrs, K-high dose sunflower oil 24 hrs and L-high dose sunflower oil 48 hrs.


A
Rapamycin 24hrs
B
Rapamycin 48hrs
C
Low Flax 24hrs
D
Low Flax 48hrs
E
High Flax 24hrs
F
High Flax 48hrs
20 µm

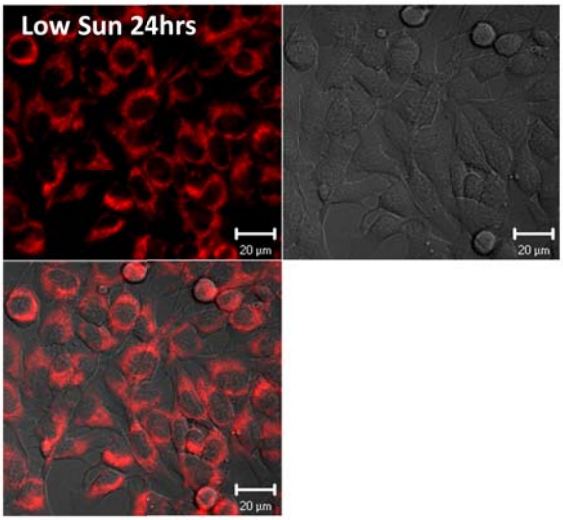
G


Control 24hrs
20 µm
20 µm
20 µm

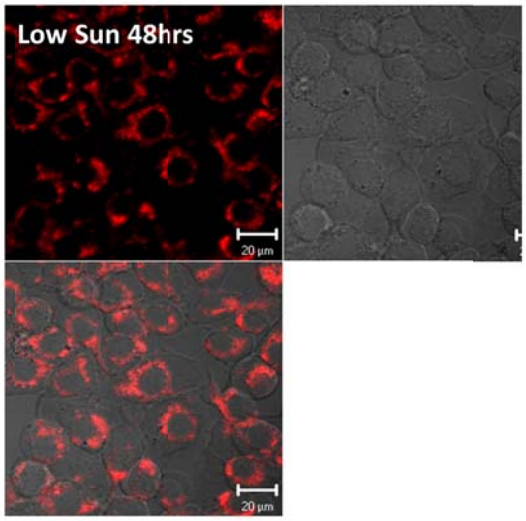
H


Control 48hrs
20 µm
20 µm

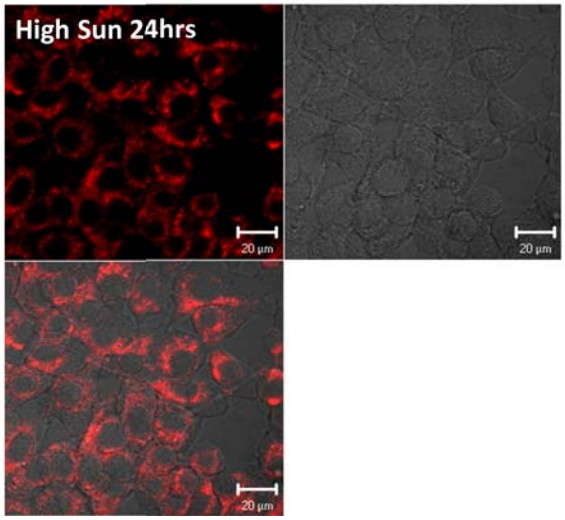
I


Low Sun 24hrs
20 µm
20 µm
20 µm

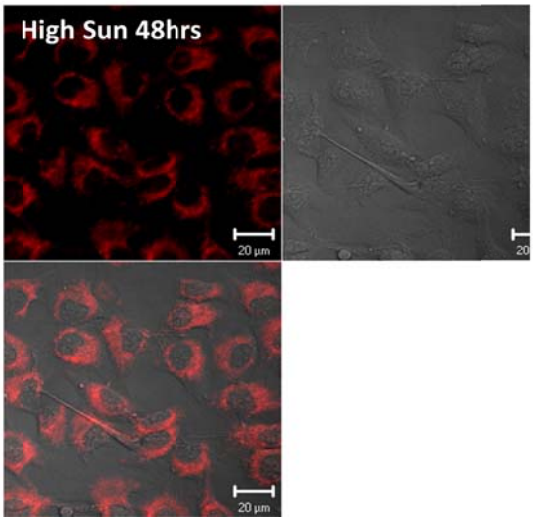
J


Low Sun 48hrs
20 µm
20 µm

K


High Sun 24hrs
20 µm
20 µm
20 µm

L


High Sun 48hrs
20 µm
20 µm

3.7 Solvent Extraction of Anti-cancer Components of Flaxseed Oil

A series of experiments were designed to study the chemical properties of the biologically active components of flaxseed oil. Since flaxseed oil is not soluble in media, these studies investigated the potential of culture media to act as an extraction solvent. Equal parts of flaxseed oil and culture media were mixed together and incubated for 24 hrs. Following incubation, a bottom aqueous and a top oil layer formed. These layers were used individually to treat B16-BL6 cells in the same manner as in all previous experiments. These studies showed that cells treated with the top oil layer had a significant decrease in cell growth, comparable to samples that were treated with flaxseed oil ($p < 0.01$) (Figure 14A). Cells treated with the aqueous layer showed no changes in cell growth (Figure 14B). Therefore the active components remain in the oil layer and are not extractable into the aqueous phase. DMSO, hexane, dichloromethane and 70% ethanol were used to further examine the chemical properties of flaxseed oil. Flaxseed oil was combined with each of these agents and their ability to extract chemical components into the aqueous phase were determined in subsequent cell growth experiments (Figure 15). None of the four solvents mentioned showed any additional inhibition of cell growth on B16-BL6 cells when compared to flaxseed oil only treatments. Furthermore, hexane alone was toxic to the cells and dichloromethane prevents the inhibitory effect on cell growth of treating B16-BL6 cells with flaxseed oil (Figure 15).

Figure 14 Media extraction of the anti-cancer components of flaxseed oil

The solubility of the active agents in flaxseed oil was determined by extracting the oil with tissue culture media and then testing the efficacy of each fraction on B16-BL6 cell growth. Flaxseed oil was combined with media at a 1 to 1 ratio, vortexed and allowed to separate over 24 hrs. Following the 24 hr incubation period, two defined layers formed: a top oil layer and a bottom aqueous layer. (* = $p < 0.05$, ** = $p < 0.01$) (A-comparison of high dose and low dose treatments with top oil layers, B-comparison of high dose and low dose treatments with bottom aqueous layers, C-comparison of high dose and low dose treatments of flaxseed oil, and D-Comparison of high dose concentrations of oil layer, aqueous layer and flaxseed oil treatment)

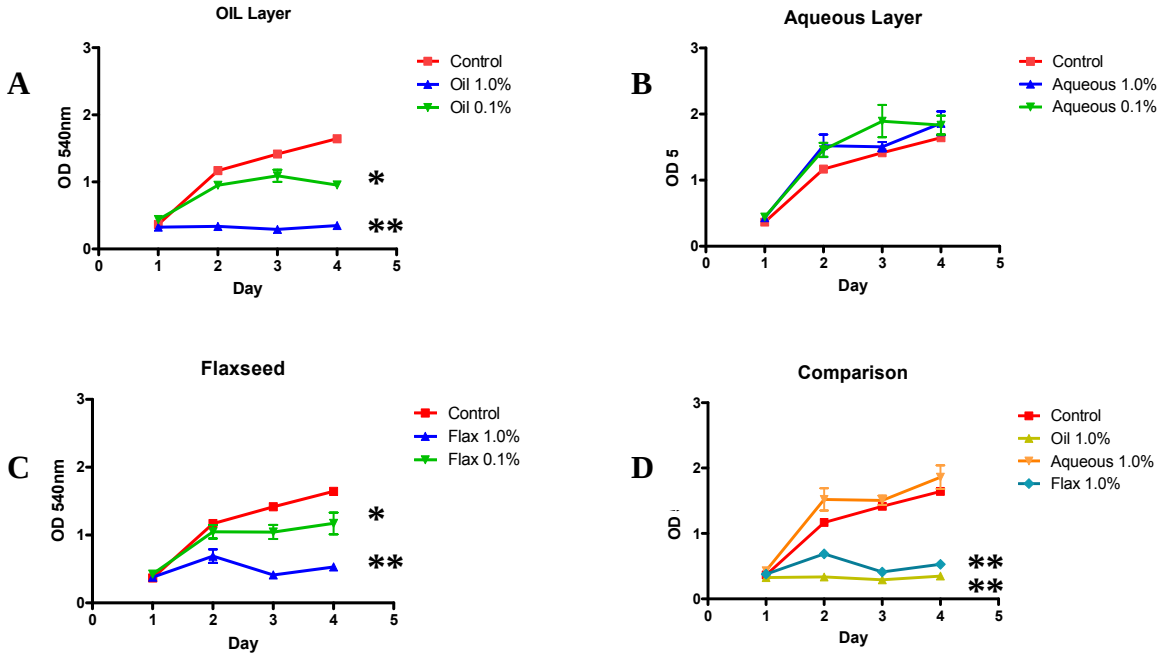
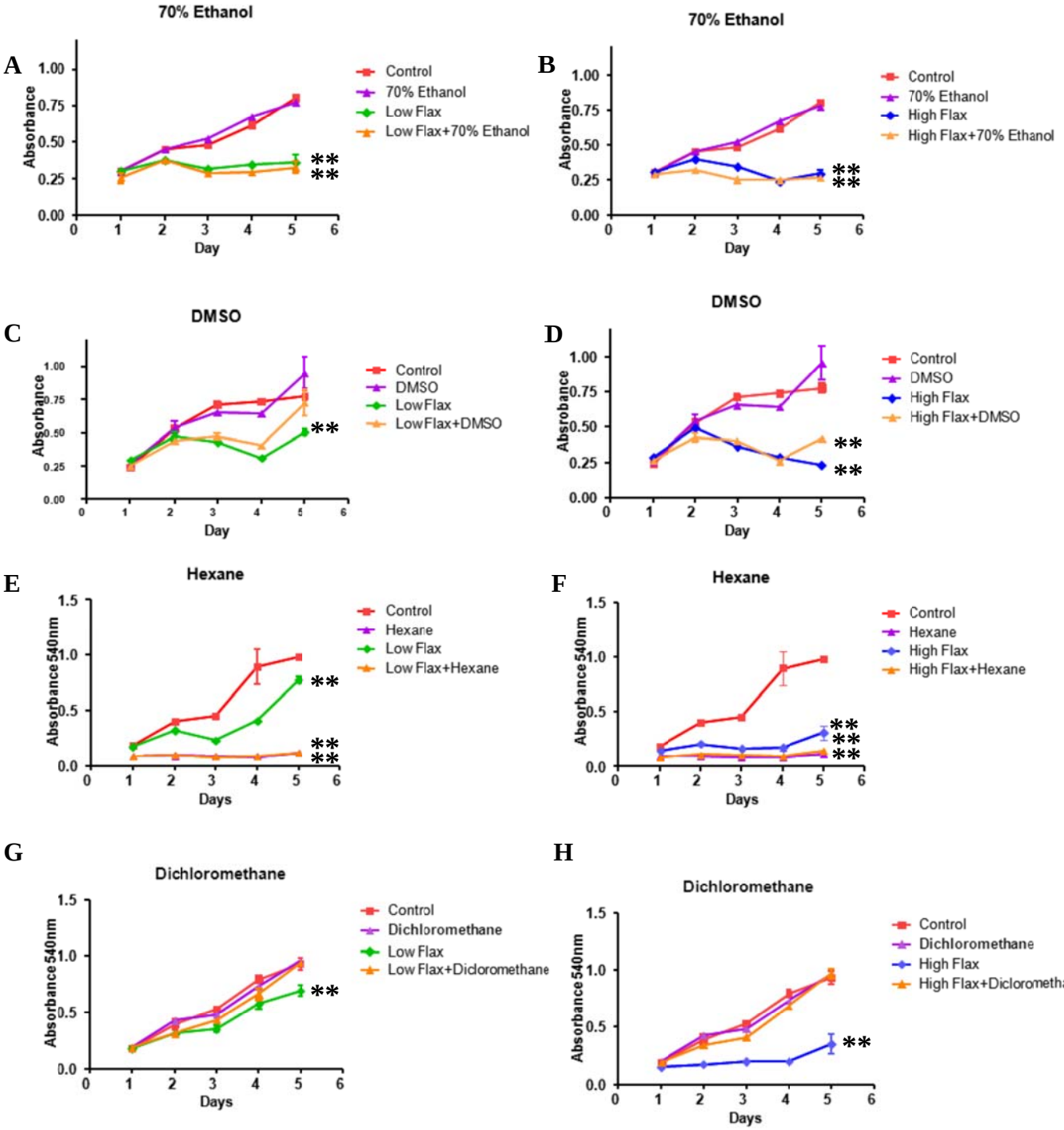

A
OIL Layer
OD 540nm
Day
Control
Oil 1.0%
Oil 0.1%
*
**
B
Aqueous Layer
OD 5
Day
Control
Aqueous 1.0%
Aqueous 0.1%
C
Flaxseed
OD 540nm
Day
Control
Flax 1.0%
Flax 0.1%
*
**
D
Comparison
Day
Control
Oil 1.0%
Aqueous 1.0%
Flax 1.0%
**
**

Figure 15 Solvent extraction of anti-cancer components of flaxseed oil

The solubility of the active agents in flaxseed oil was determined by extracting the oil with either, Dichloromethane, DMSO, hexane or 70% ethanol and then testing the efficacy of each fraction on B16-BL6 cell growth. B16-BL6 cells were treated with low dose flaxseed oil, high dose flaxseed oil, or a combination of flaxseed oil and one solvent. Treatment with pure solvent was used as a control. Flaxseed treatment was significantly different from untreated controls. There was no significant difference in flaxseed oil treatment alone and flaxseed oil plus solvent treatment for any of the four solvents used. Hexane alone and in combination with flaxseed oil was toxic to the cells. (* = $p < 0.05$, **=$p < 0.01$) A-low dose treatments and 70% Ethanol, B-high dose treatments and 70% Ethanol, C-low dose treatments and DMSO, D-high dose treatments and DMSO, E-low dose treatments and hexane, F-high dose treatments and hexane, G-low dose treatments and dichloromethane, H-high dose treatments and dichloromethane)


A
70% Ethanol
Absorbance
Day
Control
70% Ethanol
Low Flax
Low Flax+70% Ethanol
**
**
B
70% Ethanol
Absorbance
Day
Control
70% Ethanol
High Flax
High Flax+70% Ethanol
**
**
C
DMSO
Absorbance
Day
Control
DMSO
LowFlax
LowFlax+DMSO
**
D
DMSO
Absrobance
Day
Control
DMSO
High Flax
High Flax+DMSO
**
**
E
Hexane
Absorbance 540nm
Days
Control
Hexane
Low Flax
Low Flax+Hexane
**
**
**
F
Hexane
Absorbance 540nm
Days
Control
Hexane
High Flax
High Flax+Hexane
**
**
**
G
Dichloromethane
Absorbance 540nm
Days
Control
Dichloromethane
Low Flax
Low Flax+Dicloromethane
**
H
Dichloromethane
Absorbance 540nm
Days
Control
Dichloromethane
High Flax
High Flax+Dicloromethan
**

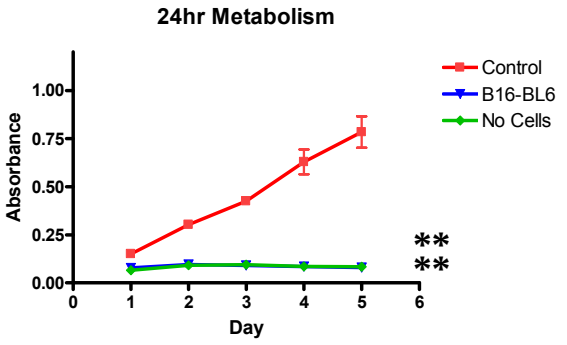
3.8 Flaxseed Oil Metabolism in B16-BL6 Cells

An experiment was designed to test the hypothesis that B16-BL6 cells are capable of metabolizing the omega fatty acids found in flaxseed oil and make the flaxseed oil less effective in causing B16-BL6 cell death. Cells were treated as usual with high dose flaxseed oil (9 µl/ml) in large tissue culture plates and incubated for several days (Figure 16). Tissue culture plates containing flaxseed oil in media only, and hence no B16-BL6 cells, were labelled "No Cells" for this experiment. Tissue culture plates containing media but no B16-BL6 cells were also placed in incubators and used as controls and were labelled "Control" for this experiment. At timed intervals, the media from each condition was collected and used to treat a set of 5 B16-BL6 96 well plates. These 96-well plates were then used for a new 5 day experiment. Over time, 96-well plates cells that were treated with flaxseed oil media that had been previously exposed to cells, had significantly less inhibition in cell growth at 120 hrs than cells that were treated with flaxseed oil containing media that was in empty tissue culture plates and left in the incubator ($p < 0.05$) (Figure 16E). This finding supports the idea that B16-BL6 cells are able to metabolize flaxseed oil and utilize as well as deplete the active anti-cancer components. To further investigate this finding, GC/MS was performed on B16-BL6 cell membranes to determine fatty acid composition following flaxseed oil treatment (Figure 17). The results showed that B16-BL6 cells treated with flaxseed oil had significant increases in polyunsaturated fatty acids (PUFA) and decreased amounts in monounsaturated fatty acids (MUFA) in the 48 hr samples when compared to untreated controls ($p < 0.05$) (Figure 17A). Cells treated with sunflower oil showed comparable levels of both PUFA and MUFA to the untreated controls at 48hrs (Figure 17A). This

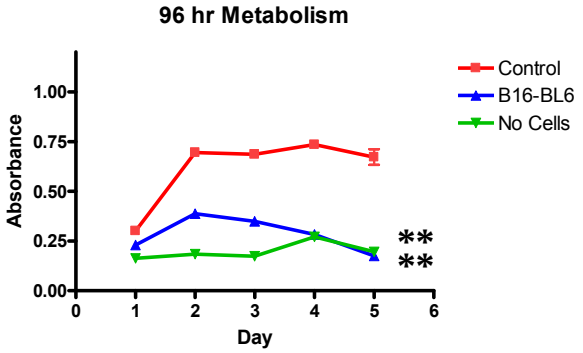
Figure 16 Omega fatty acid metabolism of flaxseed oil by B16-BL6 cells

Media containing flaxseed oil was placed in empty tissue culture plates or in tissue culture plates plated with B16-BL6 cells and incubated at 37ºC. Supernatant from these plates were collected and used for the treatment of five 96-well plates containing B16-BL6 cells. MTT survival assays were performed on these 96-well plates over 5 days. At the 120 hr timepoint, growth of B16-BL6 cells were significantly different between cells treated with flaxseed oil incubated on B16-BL6 cells and the flaxseed oil that was incubated on an empty plate. (* = $p < 0.05$, ** = $p < 0.01$)

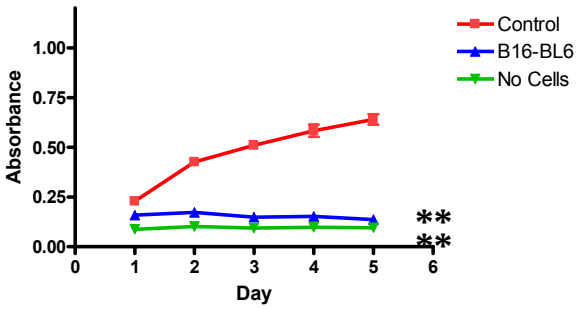
A


24hr Metabolism
Absorbance
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
Day
Control
B16-BL6
No Cells
**
**

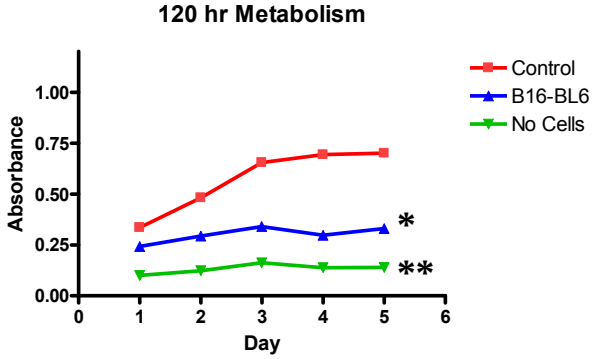
D


96 hr Metabolism
Absorbance
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
Day
Control
B16-BL6
No Cells
**
**

B


48 hr Metabolism
Absorbance
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
Day
Control
B16-BL6
No Cells
**
**

E


120 hr Metabolism
Absorbance
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
Day
Control
B16-BL6
No Cells
*
**

C

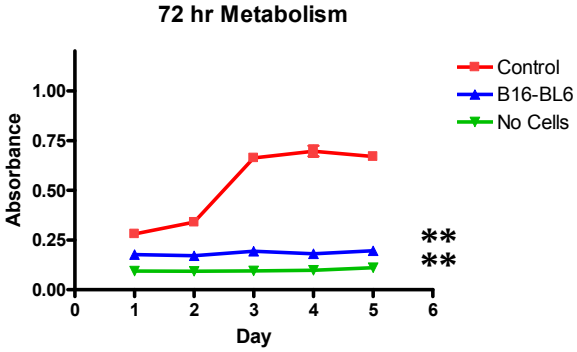
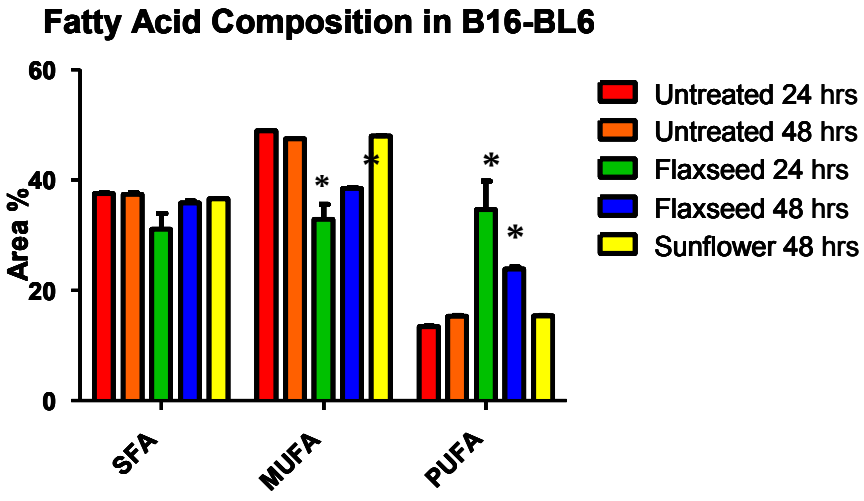

72 hr Metabolism
Absorbance
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
Day
Control
B16-BL6
No Cells
**
**

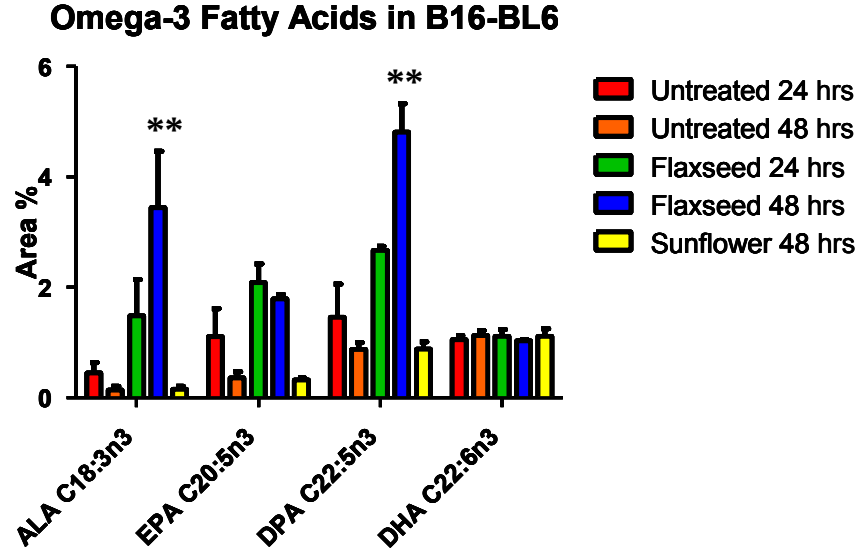
Figure 17 GC/MS determination of omega fatty acid composition of cell membranes of B16-BL6

Cell membranes of B16-BL6 treated with flaxseed oil or sunflower oil were collected and sent for GC/MS analysis at the CCARM laboratory in Winnipeg, Manitoba. Analysis determined levels of saturated fatty acids (SAF), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) (A), as well as levels of specific omega-3 (B) and omega-6 fatty acids (C). (* = $p < 0.05$, ** = $p < 0.01$)

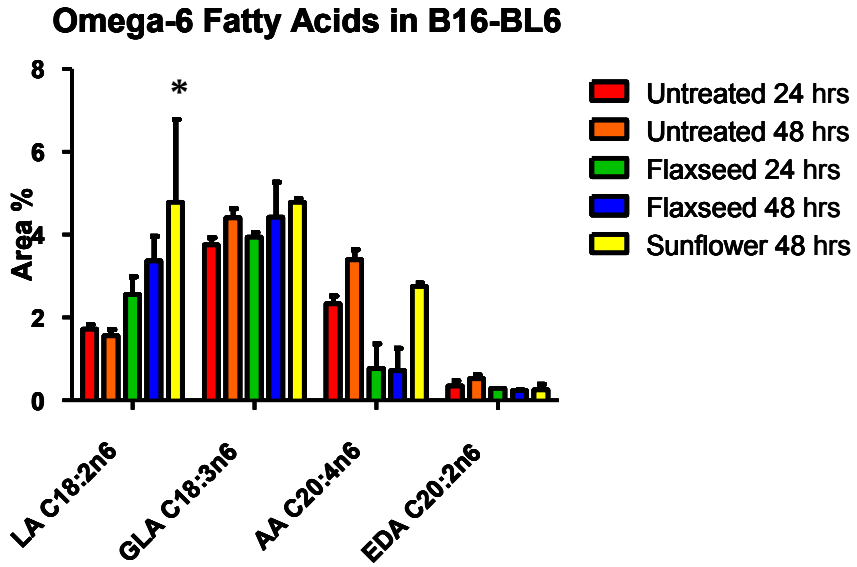
A


Fatty Acid Composition in B16-BL6
Area %
60
40
20
0
SFA
MUFA
PUFA
*
*
*
*
Untreated 24 hrs
Untreated 48 hrs
Flaxseed 24 hrs
Flaxseed 48 hrs
Sunflower 48 hrs

B


Omega-3 Fatty Acids in B16-BL6
Area %
6
4
2
0
ALA C18:3n3
EPA C20:5n3
DPA C22:5n3
DHA C22:6n3
**
**
Untreated 24 hrs
Untreated 48 hrs
Flaxseed 24 hrs
Flaxseed 48 hrs
Sunflower 48 hrs

C


Omega-6 Fatty Acids in B16-BL6
Area %
8
6
4
2
0
LA C18:2n6
GLA C18:3n6
AA C20:4n6
EDA C20:2n6
*
Untreated 24 hrs
Untreated 48 hrs
Flaxseed 24 hrs
Flaxseed 48 hrs
Sunflower 48 hrs

experiment also looked at levels of specific omega-3 and omega-6 fatty acids present in all of the cell membranes of B16-BL6 cells (Figure17B and C). Cells that were treated with flaxseed oil had significantly higher levels of omega-3 fatty acid alpha linolenic acid and docosapentaenoic acid when compared to the untreated controls and sunflower treated cells at 24 hrs ($p < 0.01$) (Figure 17B). Sunflower oil treated cells at 48 hrs had significantly higher levels of linoleic acid present in the cell membranes when compared to untreated controls ($p < 0.05$), but had similar amounts of all other omega fatty acids as compared to the untreated 48 hr controls (Figure 17C).

3.9 Flaxseed Oil Enhances Taxol-Mediated Cell Death in B16-BL6 Cells

B16-BL6 cells were treated with flaxseed oil in combination with the chemotherapeutic agent taxol (Paclitaxel®). Cells were plated in mini plates and incubated overnight at 37ºC to allow adherence. Following incubation, B16-BL6 cells were treated with flaxseed oil, taxol or a combination of flaxseed oil and taxol (Figure 18). The taxol concentrations included a high dose of the standard pharmacological concentration of 10^{-5}M and decreased by 1000x folds to the lowest concentration of 10^{-9}M. After 24 hrs, cells were photographed to determine changes in cell morphology. Cells treated with just flaxseed oil or taxol showed rounder cells and apoptotic bodies, indicative of cell death. These morphological changes are more apparent at high dose concentrations of either flaxseed or taxol. B16-BL6 cells treated in combination with flaxseed oil and taxol had the largest morphological changes (Figure 18). Cells that were treated with high dose flaxseed oil and low dose 10^{-9}M taxol appeared to have increased apoptotic bodies present than those cells treated with 10^{-9}M taxol alone (Figure 18). Flaxseed oil treatment in combination with low dose 10^{-8}M taxol was further investigated by MTT analysis (Figure 19). Cells treated with a combination of low dose flaxseed oil and low dose taxol had significantly more cell death than cells treated with only low dose flaxseed oil or low dose taxol ($p < 0.01$) (Figure 19A). These findings suggest that supplementing lower concentrations of taxol with low dose flaxseed oil can achieve similar effects on cell death than the high concentrations of taxol alone. Furthermore, the supplementation of the natural product flaxseed oil may be capable of reducing the harsh side effects caused by the administration of high concentrations of taxol, while still obtaining the same anti-cancer effectiveness just at lower doses.

Figure 18 Flaxseed oil enhances taxol-mediated cell death

Cells were plated in mini plates and treated with increasing concentrations of either flaxseed oil, taxol, or a combination of both. Taxol concentrations consisted of 10^{-9}M, 10^{-8}M, 10^{-7}M, and 10^{-6}M. Flaxseed oil concentrations consisted of the standard low dose flaxseed oil (3 µl/ml of media) and high dose flaxseed oil (9 µl/ml of media). Cells were photographed using the Fluorchem 100 microscope.

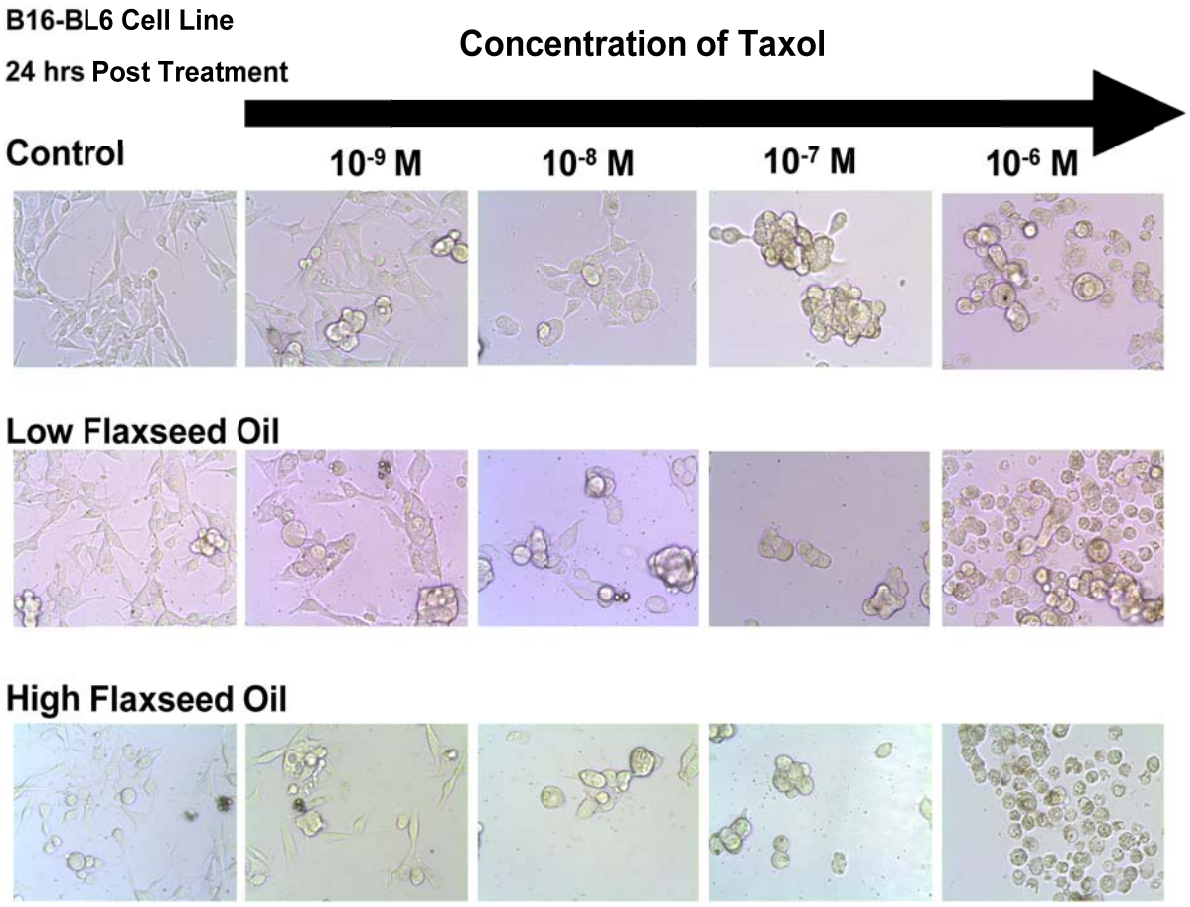
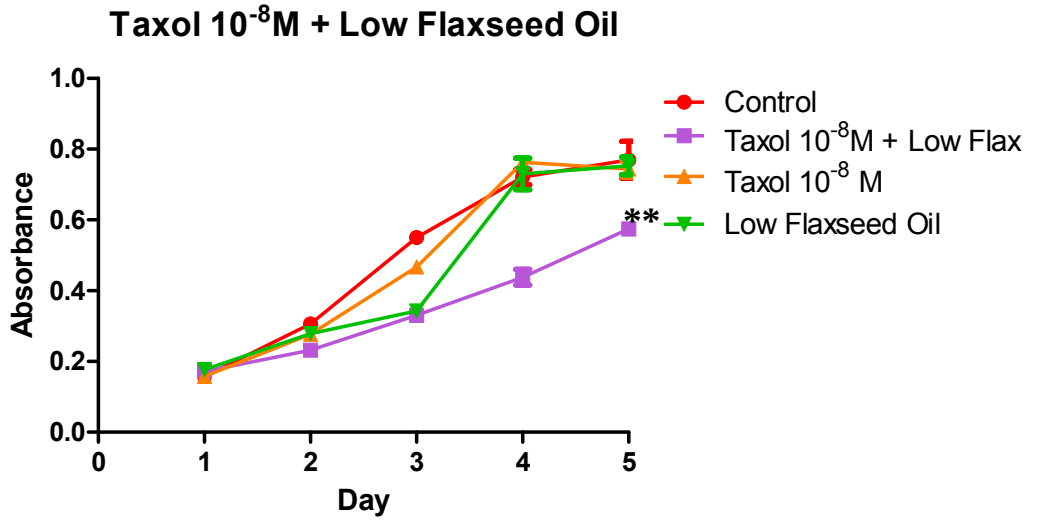

B16-BL6 Cell Line
24 hrs Post Treatment
Concentration of Taxol
Control
10-9 M
10-8 M
10-7 M
10-6 M
Low Flaxseed Oil
High Flaxseed Oil

Figure 19 Flaxseed oil enhances low dose taxol-mediated cell death

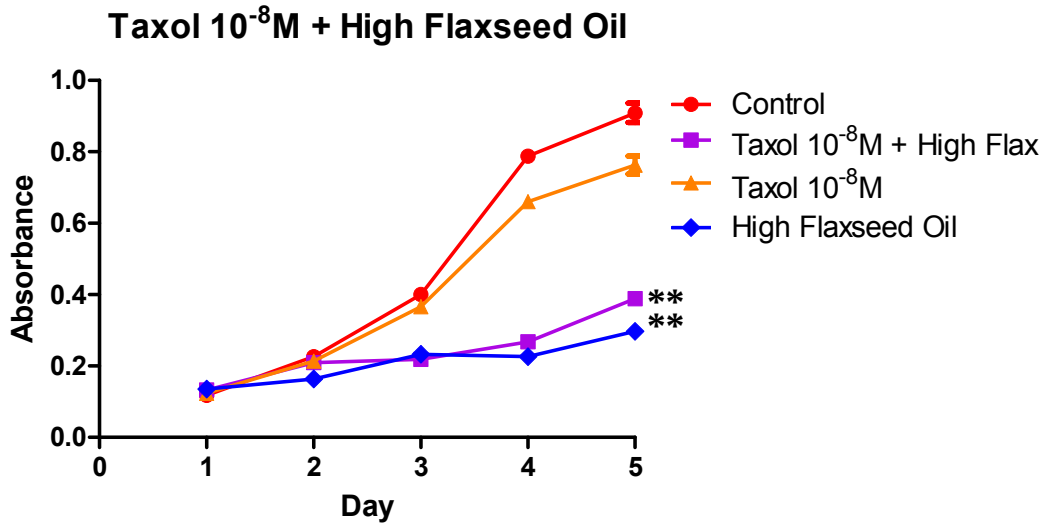
Cells were plated in 96-well plates and treated with low doses of 10^{-8}M concentrations of Paclitaxel in the presence and absence of flaxseed oil. Each day of treatment, for 5 days, the number of cells in each well of the plate was determined using the MTT assay.

(** = $p < 0.01$)

A


Taxol 10-8M + Low Flaxseed Oil
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Day
**
Control
Taxol 10-8M + Low Flax
Taxol 10-8 M
Low Flaxseed Oil

B


Taxol 10-8M + High Flaxseed Oil
Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Day
**
**
Control
Taxol 10-8M + High Flax
Taxol 10-8M
High Flaxseed Oil

4.0 Discussion

4.1 Flaxseed oil and Effects on B16-BL6 Cells

The results of these experiments support the idea that flaxseed oil affects cell growth of the aggressive murine melanoma cell line, B16-BL6. Many of the individual components of flaxseed oil have been previously investigated in *in vitro* experiments[22,23], however, our studies are the first to look at the effects of flaxseed oil as a whole, and to further investigate the metabolism of flaxseed oil following its exposure to cells in an *in vitro* model. Dr. Thompson and her laboratory at the University of Toronto are at the forefront of flaxseed research using *in vivo* models. Dr. Thompson has previously shown that nude athymic mice fed a diet supplemented with either 5% or 10% flaxseeds showed inhibition of the initiation stages of carcinogenesis[25]. Dr. Thompson's laboratory focuses on lignans (such as SDG), which are the second largest component of flax seeds following the omega fatty acids. Studies involving lignans are focused around their phytoestrogenic properties and their potential to influence estrogen sensitive cancers, including breast cancers. Since phytoestrogens bind to estrogen receptor sites, phytoestrogens have the ability to block the interaction between estrogen molecules and receptors[25]. This blockage enables phytoestrogens to protect woman from estrogen promoted cancers[25]. Lignans however are not found in significant amounts in flaxseed oil. Therefore studies which focus solely on lignans may not be describing the true properties of flaxseed oil. This idea is further supported by our previous studies that have looked at the effects of a fabricated "flaxseed oil". Omega fatty acids and lignans were combined to a 1 to 1 ratio (ALA, LA, GLA, EPA, DHA, ED and ET)[38]. Components were then dropped out or added into the mixture, and cells counts were determined using

the Trypan Blue Survival Assay[38]. Although this study has shown that certain components may have larger affects than others, the strongest inhibition of cellular proliferation occurred when B16-BL6 cells were treated with the complete fabricated "flaxseed oil" mixture of the seven compounds each at a concentration of 10^{-5}M [38].

The current project investigates the effects of various plant oils including flaxseed, peanut, canola, sunflower, olive, sesame and grapeseed on cell growth of B16-BL6 cells. Each of these oils is composed of various amounts of omega-3, -6 and -9 fatty acids. Each of the seven oils was analyzed by GC/MS and a fatty acid profile was determined. All seven oils were then used to treat the aggressive murine melanoma cell line B16-BL6. Although omega-fatty acids were strongly present in each profile, only flaxseed oil was capable of blocking cell proliferation and inducing cellular apoptosis in B16-BL6 cells. These findings further suggest that there is an optimal mixture of omega fatty acids that is required to induce apoptosis to the extent that has been shown in this study. This conclusion is also supported by past studies that have focused on the anti-cancer activities of individual omega-3 fatty acids. Researchers have shown that in animal models that were fed diets supplemented with high amounts of the omega-3 fatty acid alpha-linolenic acid, the animals exhibited reduced growth of spontaneous and carcinogen-induced mammary tumours[57-59]. Furthermore, there have also been various studies that investigated the individual effects of treating cancer cells with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). One study examined the effects of dietary EPA and DHA and their ability to suppress tumour growth in the human cancer cell line MDA-MB-435[44]. Female athymic nude mice were fed diets that were supplemented with 4% or 8% of EPA, DHA or ALA, and compared to mice fed the

standard non-supplemented feed. Rose and colleagues found that both the 8% EPA and 8% DHA fed mice had the smallest growth of tumour implanted in the mammary fat pads[44]. These experiments show that the individual omega-3 fatty acid components found in flaxseed oil may possess some anti-cancer activity. It is our belief that the individual components of flaxseed oil may have a potential additive and/or synergistic effect when malignant cell lines are treated with flaxseed oil as a whole rather than as fractionated individual components. This effect may be the result of cellular apoptotic induction through various biological pathways.

4.2 Apoptosis and Cell Morphology

Cell morphology can often be the first telling sign that a cell is undergoing apoptosis, a type of cell suicide. Cell death can be classified as apoptosis, necrosis or autophagy[60]. Apoptosis is recognized by many morphological changes of which the most significant include plasma membrane blebbing, chromatin condensation and nuclear fragmentation[60]. The current study looked at morphological changes in B16-BL6 cells using the acridine orange and ethidium stain and fluorescence microscopy. Acridine orange and ethidium bromide are selective cationic dyes that interact with DNA through intercalation. Acridine orange is cell membrane permeable and stains all cells, while ethidium bromide only stains cells when the plasma membrane has been compromised such as in necrotic or late apoptotic cells. Acridine orange fluoresces green while ethidium bromide fluoresces red. This study showed increased morphological changes in B16-BL6 cells following treatment with the positive control cyclohexamide and the flaxseed oil-treated cells at both high and low doses and time points greater than 24 hr

when compared to untreated controls. Flaxseed oil-treated B16-BL6 cells had increased acridine orange and ethidium bromide staining, as well as membrane blebbing as compared to control. The flaxseed oil-treated B16-BL6 cells did not however have significant saturation points of ethidium bromide staining until longer time points following treatment. These results indicate that the cells are not necrotic, but instead are undergoing apoptosis. The current study is supported by previously published findings. Song *et al.* [61] detected morphological changes in both pancreatic cell lines, SW1990 and PANC-1, following treatment with docosahexaenoic acid. Furthermore, treatment with the omega-6 fatty acid arachidonic acid did not alter the morphology of the pancreatic cells[61]. Flaxseed oil induced apoptosis in B16-BL6 cells which is an important finding. It supports the hypothesis that the cells are receiving a signal from the flaxseed oil and biochemically inducing cell destruction rather than swelling up and consequently bursting as would be seen in necrotic cells.

4.3 TUNEL and DNA Damage

DNA is severely damaged during the process of apoptosis. Although there are numerous morphological characteristics of apoptotic cells, DNA fragmentation is not limited to histologically defined apoptotic cells, but can also be present in cells considered to be morphologically intact[62]. It is therefore also important to investigate apoptosis using techniques other than cellular morphology stains. TUNEL stain detects DNA damage *in situ* by labelling dUTP-biotin nicked ends using the enzyme terminal deoxynucleotidyl transferase[62]. The current study shows increased TUNEL staining in the positive control, camptothecin-treated cells, as well as in the flaxseed oil treated cells.

Treatment with flaxseed oil at both low and high doses, and at both the 24 hr and 48 hr treatment time points, increased the number of TUNEL stained cells compared to untreated and sunflower-treated cells. The cells treated with high dose of flaxseed oil for 48 hrs had very bright staining. This is an indication of increased DNA breaks often associated with late phase apoptosis. These findings are similar to other published results that have looked at the ability of treatment with individual omega-3 fatty acids to induce apoptosis. Song *et al.*[61] found docosahexaenoic acid treated SW1990 pancreatic cancer cells after 24 hrs had an increased number of TUNEL-positive cells when compared to the untreated controls and to the omega-6 fatty acid treated cells. Morin *et al.*[63] also found similar results when studying the effect of docosapentaenoic acid, an omega-3 fatty acid that has received very little attention on cell death. Morin's research group showed that in HCT116 tumour-bearing nude mice, orally administered docosapentaenoic acid monoglyceride, showed an increase in apoptotic cancer cells[63]. The apoptotic index was calculated at 48 ± 2.5% in tumour sections derived from docosapentaenoic acid monoglyceride treated mice and 4.5 ± 0.9% in tumour sections from control mice[63]. The ability of cancer cells to escape apoptosis is a hallmark characteristic of cancer under normal conditions. Therefore any natural products that have the ability to activate apoptotic pathways in cancer cells are of high importance in developing anti-cancer therapeutics.

4.4 Annexin V-FITC and Apoptosis

The current studies have determined that flaxseed oil induced cell death in B16-BL6 cells through the activation of the apoptotic pathway. There are two pathways that

are involved in the apoptotic process, the intrinsic and the extrinsic pathway. The current study has examined apoptosis in B16-BL6 cells following flaxseed oil treatments using DNA fragmentation gels, fluorescence microscopy and flow cytometry. Previous work on this project showed that flaxseed oil activated apoptosis through the extrinsic pathway. Western blot analysis showed caspase cleavage following 24 hr treatment with flaxseed oil[38]. Our previous studies also showed increased DNA fragmentation in flaxseed oil-treated cells compared to untreated controls (Appendix C)[38]. The current studies have further investigated the induction of apoptosis following flaxseed oil treatment. Flow cytometry was used to look at changes in Annexin V staining in B16-BL6 cells at 24 hrs, 48 hrs and 72 hrs, following flaxseed oil treatment. Under normal physiological conditions, phosphatidylserines are located on the cytosolic side of the plasma membrane. Following physiological triggers and the induction of apoptosis, phosphatidylserines are translocated to the outer edge of the cell membrane[64,65]. This shift in phosphatidylserines from the inner membrane to the outer membrane signals to surrounding phagocytic cells to target the cell for phagocytosis and destruction. This translocation step also allows for Annexin V labelling, and cells currently undergoing apoptosis can be detected by FACS analysis using the Annexin V-FITC conjugate. The current study shows increased detection of Annexin V-FITC binding to cyclohexamide-treated cells, the positive control, and to the flaxseed oil-treated cells at both doses (low and high) as well as at all time-points (days 1, 2 & 3) when compared to untreated controls and sunflower oil-treated cells. These findings are consistent with previously published works. Researchers have previously shown that omega-3 fatty acid-rich oils, such as flaxseed oil, are able to provide protection against the progression of colon

cancers, whereas omega-6 fatty acid-rich oils such as sunflower oil, may in contrast promote tumourigenesis in these same colon cancers[65]. Bommareddy *et al.* [65] showed by FACS analysis using Annexin V staining, that alpha-linolenic acid at concentrations of 800 μM and 1000 μM significantly induced apoptosis in the human colon adenocarcinoma cells, Caco-2. Bommareddy *et al.* [65] further showed that lower concentrations (500 μM) of alpha-linolenic acid could also induce similar levels of apoptosis in Caco-2 cells, if administered in combination with 75 μM of either enterolactone or enterodiol, two lignans found within flaxseed oil. It is suggested that the higher concentrations of alpha linolenic acid that were required to induce apoptosis to the same degree as was accomplished by sole treatment with enterodiol or enterolactone, may in part be due to alpha-linolenic acid being metabolized into its longer chain counterparts, eicosapentaenoic acid and docosahexaenoic acid[65]. As previously mentioned, this conversion is an inefficient process and therefore larger amounts of alpha-linolenic acid are required to produce sufficient amounts of the active metabolites.

4.5 PI Stain and the Cell Cycle

FACS analysis was further used to determine the effects of flaxseed oil on cell division and the cell cycle in B16-BL6 cells. Propidium iodide, a stoichiometric stain, was used to determine DNA content and hence enable B16-BL6 cells to be classified into phases of the cell cycle based on DNA content. This study showed that B16-BL6 cells stained with propidium iodide had significant changes in cell populations for the various phases of the cell cycle in both the positive control-treated cells, which included camptothecin-treated cells, as well as the flaxseed oil-treated cells when compared to the

untreated cells. FACS analysis of B16-BL6 cells at 24 hrs and 36 hrs following flaxseed treatments, showed increased cells numbers in sub G1 phase of the positive control, camptothecin-treated cells, as well as cells treated with both doses of flaxseed oil when compared to untreated control cells. These findings are supported by previously published work. Song *et al.*[61] have shown the effect of docosahexaenoic acid on the pancreatic cell line SW1990. Their studies showed that docosahexaenoic acid resulted in an increased sub-G1 population for SW1990 cells. Furthermore, the effect of docosahexaenoic acid treatment was shown to be dose-dependent[61]. Although these findings support our current studies, there are still important differences to point out. For example, the majority of research currently takes place using complex mixtures of omega-3 fatty acids, while this study focuses on one individual member of the omega-3 fatty acid family, docosahexaenoic acid, and its effect on the cell cycle. Our studies are complicated by using flaxseed oil as a whole. Dekoj *et al.*[66] is one of few laboratories to investigate the effects of omega-3 fatty acids in combination and their effects on cancer cell growth. The Dekoj research group uses omega-3 fatty acid and omega-6 fatty acid emulsions purchased from Fresenius-Kabi in Bad-Homburg, Germany[66]. The omega-3 fatty acid emulsion, Omegaven®, and the omega-6 fatty acid emulsion, Lipovenoes®, are clinically used for parenteral nutrition[66]. MIA PaCa-2, human pancreatic ductal adenocarcinoma cells, showed cell cycle arrest at the G2/M interface when treated with the omega-3 fatty acid emulsion for 24 hrs[66]. Similar to our findings, Dekoj *et al.* [66] showed increased sub-G1 populations in MIA PaCa-2 cells at 48 hrs post omega-3 fatty acid emulsion treatments. Further supporting our findings, cells treated with omega-6 fatty acid emulsion showed no differences at both 24 hr and 48 hr time points when

compared to the untreated controls. This study also showed a dose-dependent response[66]. Since most people have a tendency to take omega-3 supplements which contain more than one omega-3 fatty acid, it is beneficial to look at the additive and synergistic effect that multiple omega-3 fatty acids may have on cellular process. Since flaxseed is an excellent source of omega-3 fatty acids, these studies may provide insight into these complex relationships.

4.6 Mitochondrial and Lysosomal Changes in Flaxseed Oil Treated Cells

It has been shown that apoptosis can be induced in cells via two pathways[47-49]. The external pathway requires a ligand to bind to a death receptor on the cell membrane, while the internal pathway induces apoptosis following destabilization of the mitochondrial membrane potential[47-49]. Our previous results have shown that flaxseed oil is capable of inducing apoptosis through the external pathway[38]. The current study confirmed by mitotracker red stain that cells treated with flaxseed oil can also induce apoptosis through mitochondrial destabilization and hence the internal apoptotic pathway. Following treatment with flaxseed oil, cells had significantly less mitotracker red stain when compared to the untreated controls. The mechanism by which omega-fatty acids induce apoptosis in cancer cells is poorly understood. It has been suggested that omega-3 fatty acids negatively affect mitochondria by increasing the levels of reactive oxygen species (ROS) within the cells[41]. It has also been suggested that ROS may be capable of inducing apoptosis in cells through the permeabilization of lysosomal membranes[41]. Our studies however did not show any changes in lysosome formation following flaxseed oil treatment when compared to the untreated controls. Therefore, further studies which

focus on mitochondrial membrane destabilization following flaxseed oil treatment are required.

4.7 Omega-3 Fatty Acid Metabolism

Omega-3 fatty acids have been showed to have multiple cellular functions. Changes in environmental levels of omega-3 fatty acids have the ability to greatly influence membrane fluidity and consequently can affect biological processes that take place either within, or close to cell membranes[67]. These processes can include enzymatic functions, receptor-ligand interactions, cell-cell interactions and nutrient transport through the membrane[67]. There have been two proposed hypotheses on how omega-3 fatty acids are able to disperse themselves through the cell membrane. The first suggests passive diffusion, where fatty acids use their hydrophobic tail to penetrate the outer leaflet of the cell membrane[68]. The omega-3 fatty acid is then able to rotate in a slow moving manner, dispersing itself within the bilayer, and ultimately finishing up on the inner leaflet of the cell membrane[68]. This displacement is regulated by both the physical properties of the cell membrane, as well as the structural properties of the individual omega-3 fatty acid[68]. The second mechanism is described as active transport which is mediated through plasma membrane fatty acid-binding proteins, fatty acid translocases, and fatty acid transport proteins[68]. It is also widely accepted that omega-3 fatty acids present in the cell membrane are enzymatically metabolized in response to certain physiological triggers. Cyclooxygenases and lipoxygenases metabolize omega fatty acids to form prostaglandins and leukotrienes, respectively[69]. Although the current study does not attempt to identify which of the proposed mechanisms mediates how the flaxseed oil

drug treatment is transported into the cell membrane or which enzymes are utilized for its metabolism, our results from the metabolism experiment do support the notion that flaxseed oil is capable of incorporating itself into the membrane and can then be metabolized into its longer chain counterparts in the cell membrane of B16-BL6 cells. Fatty acid metabolism has been investigated in hepatocytes by Alhazzaa *et al.* [69]. This group found that alpha-linolenic acid bioconversion to the longer chain polyunsaturated fatty acids, eicosapentaenoic acid and docosapentaenoic acid, can be attributed to various factors including enzyme affinity, substrate availability as well as transcriptional factors[69]. Furthermore, direct competition between omega-3 fatty acids can also affect bioconversion efficiencies. It has been suggested that metabolism of alpha-linolenic acid competes for enzymes, and therefore limits docosahexaenoic acid metabolism, and consequently formation of its longer metabolite docosapentaenoic acid in hepatocyte membranes[70,71]. Our current study has not further investigated this limitation. However, our studies did show that docosapentaenoic acid was present in B16-BL6 cell membranes in significantly higher quantities at 3 days, following flaxseed oil treatments, as compared to untreated controls. Since a significant increase in cell death can be identified at day 3, it may be plausible that alpha-linolenic acid metabolism may require 3 days, in order to produce sufficient amounts of omega-3 fatty acids metabolites which are capable of inducing apoptosis. Alhazzaa *et al.*[69] showed that eicosapentaenoic acid levels could be detected in FaO cells only after 3 days following administration of 50 μM of alpha-linolenic acid. It was further concluded that following day 3, significant increases in docosapentaenoic acid and docosahexaenoic acid levels could be detected in the cell membranes of FaO cells, while there was a notable reduction in eicosapentaenoic acid

levels[69]. This finding suggests that periods of longer than 3 days are required for alpha-linolenic to be converted to its long chain metabolite, docosahexaenoic acid.

4.8 Flaxseed Oil and Chemotherapeutic Agents

Omega-3 fatty acids have been described as extraordinary compounds that have the ability to slow cancer cell proliferation and increase chemotherapy effectiveness, all while calming the common side effects of chemotherapies and cancer progression[72]. It is believed that omega-3 fatty acids have the ability to alter toxicities and/or the activity of chemotherapies that are administered to cancer patients undergoing treatment. As previously mentioned, omega-3 fatty acids alter membrane fluidity and membrane-mediated functions. The current study showed that flaxseed oil treatment of B16-BL6 cells in combination with paclitaxel-enhanced cell death. This finding is supported by a newly synthesized chemotherapeutic drug. DHA-paclitaxel has been developed by covalently conjugating docosahexaenoic acid to the paclitaxel molecule. A Phase 3 study in patients provided supportive data that this new DHA-paclitaxel drug has increased anti-cancer activity and improved therapeutic applications[73]. The efficiency of using omega-3 polyunsaturated fatty acids in combination with chemotherapeutic drugs is dependent on the omega-3 fatty acid, the chemotherapeutic drug, and the type of cancer that is being treated[73]. Farhmann *et al.*[74], showed that eicosapentaenoic acid and docosahexaenoic acid were effective at increasing doxorubicin cytotoxicity in B-leukemic cells. In MDA-MB-231 breast cancer cells, docosahexaenoic acid decreased cell viability to 21% when administered with doxorubicin[25,26]. Doxorubicin alone decreased cell viability to only 54%. The suggested mechanism by which

docosahexaenoic acid increases doxorubicin cytotoxicity has been credited to its effect on generating lipoperoxides[25,26]. Further research on omega-3 fatty acids and their interactions with chemotherapeutic drugs is needed to determine their full potential.

4.9 Future Directions- Cell Signaling

The effects of flaxseed oil are complicated by the combination of the individual effects of each omega-3 fatty acid. In order to rationalize the effects of flaxseed oil treatment, the activation of signalling pathways as measured by transcription factor activation were assayed. The level of transcription factor activation measured as the level of luciferase production from specific promoter elements was determined in flaxseed oil treated and untreated transfected cells. The luciferase signaling assay was not conclusive in determining which pathway or pathways are activated and/or inhibited in B16-BL6 cells following flaxseed oil treatment, and further experiments are necessary to identify key signaling pathways. Previously published work has shown certain pathways to be altered in the presence of omega fatty acids. In hepatocarcinoma cells, eicosapentaenoic acid is credited with inhibition of cell proliferation, whereas docosahexaenoic acid is credited with the induction of apoptosis[75]. The induction of apoptosis following treatment of docosahexaenoic acid is suggested to include the ability to modify expression levels of the Bcl-2 protein family[76]. Docosahexaenoic acid is capable of increasing levels of pro-apoptotic proteins Bak and Bcl-xS and additionally decreasing levels of anti-apoptotic proteins Bcl-2 and Bcl-xL[76]. Researchers have furthermore shown that docosahexaenoic acid can also potentially induce cytochrome c release consequentially depolarizing mitochondrial membranes[77]. These results therefore

suggest that docosahexaenoic acid has the ability to activate apoptotic pathways. Eicosapentaenoic acid on the other hand has been credited with inhibiting cell proliferation. Sauer e*t al.*[78] showed that eicosapentaenoic acid inhibited cell proliferation in MCF-7 human breast cancer cells by binding to a cell surface inhibitory GPC FFAR protein. This binding causes an inhibition of adenylyl cyclase and subsequently a reduction of intra-tumour cAMP[78]. However, like the additive benefit of treating patients with both chemotherapies and omega-3 fatty acids, noticeable changes in signalling pathways are dependent on the cancer cell type, as well as omega-3 fatty acid dosages. Researchers have previously found varying results in cell signalling pathways due to these variables. In vascular smooth-muscles Tachjian *et al.*[79], showed that docosahexaenoic acid actually had a greater effect on cell proliferation than eicosapentaenoic acid. These differences make it important to consider each cell line individually and to test varying concentrations of omega-fatty acids, as these two factors may influence metabolism and incorporation in mammalian cells[75]. Our studies may also show insight on how the administration of combinations of omega-3 fatty acids may also be capable of varying the outcome on cell growth and cell death.

5.0 Conclusion

Although the underlying mechanisms have yet to be determined, published works show that omega-3 fatty acids are capable of prolonging survival of cancer patients by reducing the effects of cancer, and by providing relief of symptoms often associated with malignancy. Since the Western diet has a grossly imbalanced omega-3 to omega-6 fatty acid ratio, it is important to incorporate foods that are high in omega-3 fatty acids into our daily diets. Flaxseed oil is one of the highest plant sources of the omega-3 fatty acid alpha-linolenic acid. These studies have shown that B16-BL6 cells are capable of incorporating and metabolizing alpha-linolenic acid into longer chain omega-3 fatty acids in the cell membrane. Previously published work has shown the cellular changes omega-3 fatty acids can have when incorporated into the diet and/or used for treatment. Our studies heavily support these changes, including inhibition of cellular proliferation and induction of cellular apoptosis in malignant cells. Although these studies were unsuccessful in determining the exact mechanism by which flaxseed oil is capable of interacting with B16-BL6 cell membranes, we have concluded that flaxseed oil can be metabolized within the cell membrane into the longer chained omega-3 fatty acids (Figure 20). These studies have shown that following metabolism is the cell membrane, the longer chained omega-3 fatty acids induced apoptosis by cleaving caspase-3 while simultaneously destabilizing mitochondrial potential resulting in apoptosis (Figure 20). Furthermore, our studies have shown the potential for optimizing chemotherapeutic treatments using Paclitaxel® and flaxseed oil in combination. These findings should be of clinical importance and the results of these studies may provide a basis for the development of a powerful anti-cancer drug with minimal side effects. Future works

include further investigation of the metabolism of fatty acids within the cell membranes. Once the metabolism of omega-3 fatty acids within malignant cell membranes is understood, experiments focused on the interaction of specific omega-3 fatty acids with one another as well as with intracellular signalling proteins will allow for the development of a flaxseed oil based drug that can be tested *in vivo*.

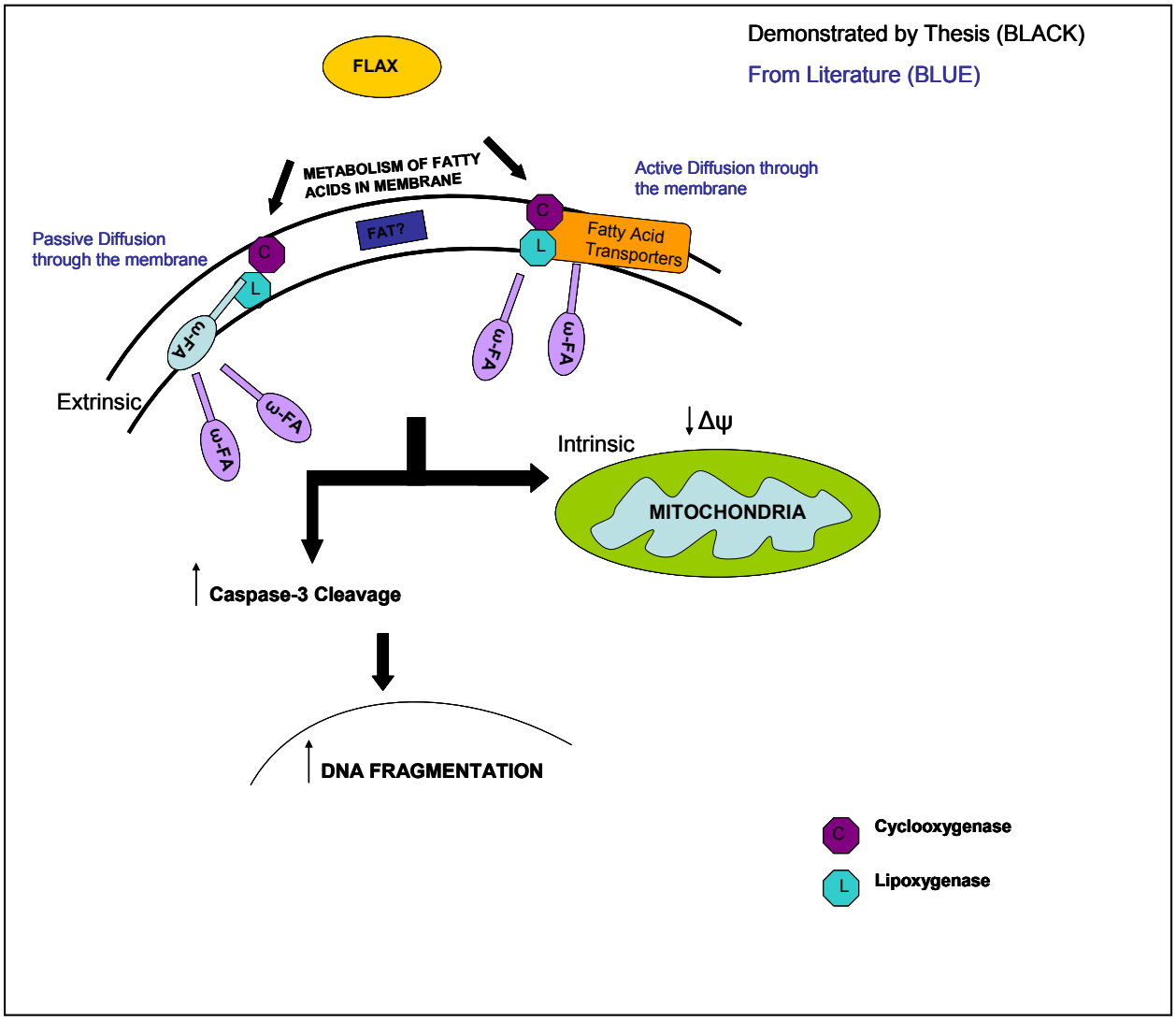


Figure 20 Proposed hypothesis of how flaxseed oil is metabolized within the cellular membrane resulting in the activation of the apoptotic pathway.

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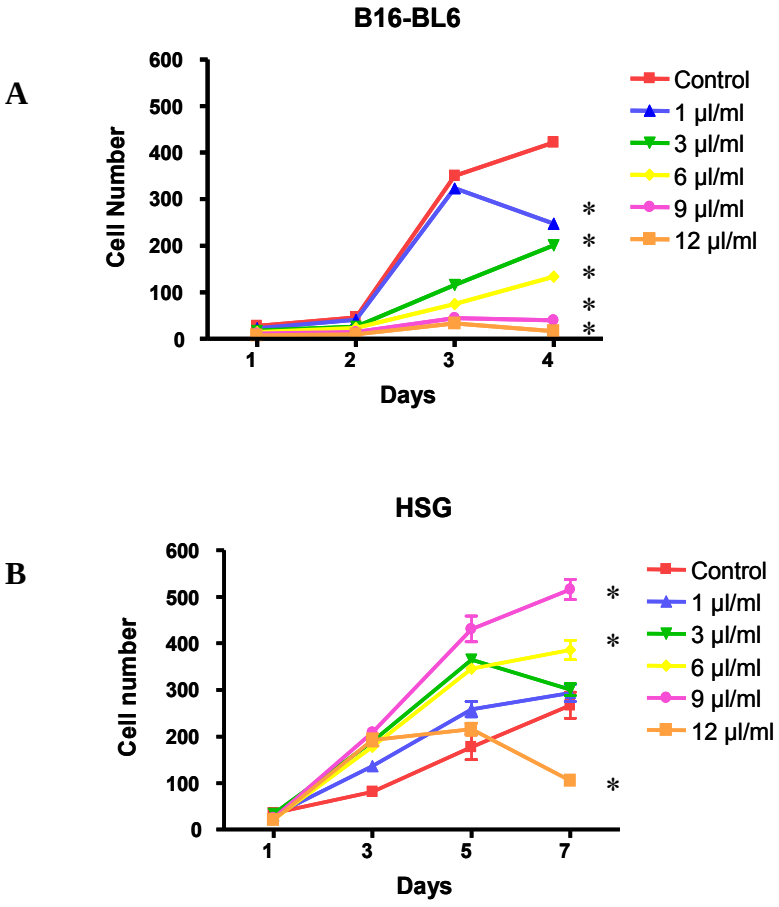
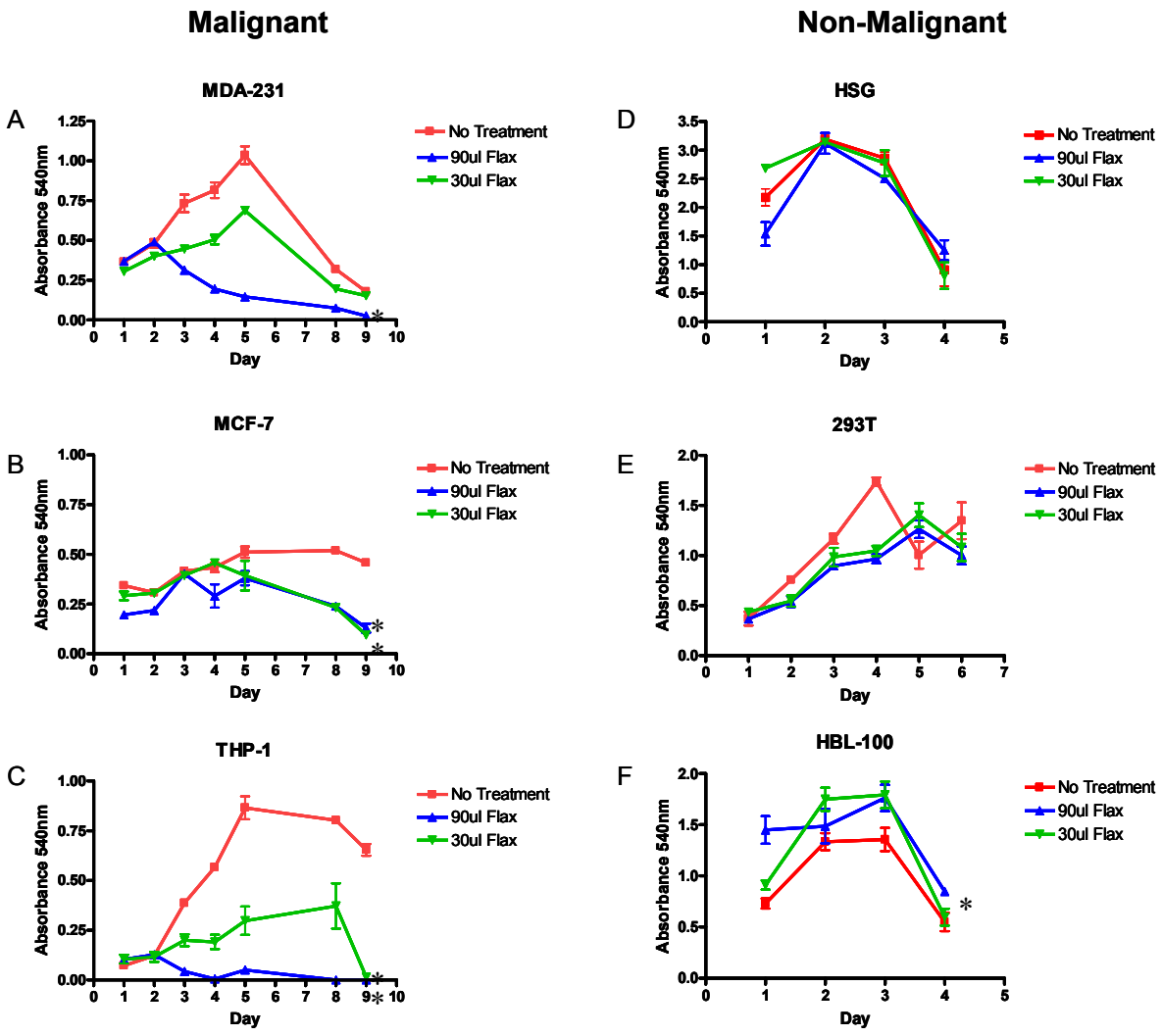
Appendix A Effect of flaxseed oil on the growth of the malignant B16-BL6 cell line and the non-malignant cell line HSG.

The change in cell number for B16-BL6 melanoma cells and HSG (human salivary gland) cells exposed to various concentrations of flaxseed oil treatments (1, 3, 6, 9 and 12 µl of flaxseed oil per millilitre of media) were determined by counting live cells after trypan-blue staining using a hemacytometer. Cells were subcultured at approximately 30% confluency one day prior to the experiment. The cells were treated with the indicated dose of flaxseed oil on day 0 and maintained for the duration of the experiment. All experiments were run in triplicate and data were analyzed for 3 experiments. A) The effects of flaxseed oil on cell growth of the aggressive murine melanoma cell line B16-BL6. B) The effects of flaxseed oil on cell growth of the non-malignant HSG cell line. (*= $p < 0.05$)



Appendix B MTT (Thiazol blue tetrazolium bromide) analysis of the cell growth of flaxseed treated malignant and non-malignant cell lines.

The viability of cultures of the malignant A) MDA-MB-231, B) MCF-7 and suspension C) THP-1 cells and the non-malignant D) HSG, E) HBL100, and F) 293T cells treated with flaxseed oil was determined using an MTT assay. The cells were treated with high dose (9 µl/ml), low dose (3 µl/ml), or culture media on day 0 and maintained for the duration of the experiment. (*= $p < 0.05$)



Appendix C DNA laddering gel of the T-lymphocyte Jurkat cell line.

The effect of treatment with flaxseed oil on apoptosis on the Jurkat T-lymphocyte cell line was examined by DNA fragmentation analysis. DNA fragmentation was analyzed in Jurkat cells treated with suspending media (lane 1), camptothecin (lane 2) or flaxseed oil (lane 3-6) for 6 h. In lanes 3 and 4 are the high and low doses of flax1 and in lanes 5 and 6 are the high and low doses of flax 2, respectively. Purified small molecular weight DNA was subjected to electrophoresis on a 2% agarose gel containing ethidium bromide.